The Effect of Cell wall Structure on Pneumococcal Virulence

Dissertation

zur Erlangung des akademisches Grades
doctor rerum naturalium
(Dr. rer. nat.)
im Fach Biologie

eingereicht an der

Mathematisch-Naturwissenschaftliche Fakultät I

der Humboldt-Universität zu Berlin

von
Diplom Biologe Florian Gehre
geboren am 09.Juli 1978 in Erlangen

Präsident der Humboldt-Universität zu Berlin
Prof. Dr. Dr. h.c. Christoph Markschies

Dekan der Mathematisch-Naturwissenschaftliche Fakultät I
Prof. Dr. Lutz-Helmut Schön

Gutachter: 1. Prof. Dr Rainer Borriss, Berlin

2. Prof. Dr Alf Hamann, Berlin

3. Prof. Dr Alexander Tomasz, New York

eingereicht: 5. Juni 2009

Datum der Promotion: 22. Oktober 2009

Abstract

Streptococcus pneumoniae (the “pneumococcus”) is a gram-positive bacterium. It is a human pathogen that leads to severe diseases such as pneumonia, sepsis, otitis media and meningitis. Being a gram-positive bacterium, it is surrounded by a thick cell wall layer. This cell wall represents the outer surface of the pathogen and therefore is the major target for the immune system during an infection. The aim of my thesis was to understand the impact of the cell wall structure on pneumococcal virulence. Particularly I was interested to test the role of acetyl-groups and choline residues of the cell wall in the pathology of the disease. Therefore I studied deacetylated and choline-free pneumococcal mutants not only biochemically *in vitro* but also in respect to their virulence in various animal models of pneumococcal disease.

In the first part of my studies I analyzed a recently discovered mutant bacterium, deficient in gene *adr*, which was described to have decreased resistance to lysozyme *in vitro*. By DNA sequence comparison and chemical analysis of highly purified cell wall I was able to identify *adr* as the structural gene of the pneumococcal peptidoglycan *O*-acetyl-transferase. Since Adr is responsible to attach *O*-linked acetyl groups to the *N*-acetyl muramic acid residues of the cell wall, *adr* mutant bacteria lack this cell wall modification. I further demonstrated that Adr does not have any impact on pneumococcal attachment to human pharyngeal cells *in vitro*. However, *adr* mutant bacteria showed a dramatic decrease in their capacity to colonize the murine nasopharynx *in vivo*. This impairment is most likely due to their enhanced sensitivity to lysozyme.

In the second part of my thesis I worked on choline residues, a structural component of the (lipo)teichoic acids of the cell wall. This part of the thesis was especially intriguing since *S. pneumoniae* has an auxotrophic requirement for this nutrient. Therefore, the recent construction of a choline-independent strain Cho^- allowed me to investigate in detail the role that this aminoalcohol plays in the virulence of the pneumococcus during meningitis and sepsis.

For the meningitis model, the choline containing strain D39Cho^- and its isogenic choline-free derivative D39Cho^-licA64 (each expressing the capsule polysaccharide 2) were introduced intracisternally into 11 days old Wistar rats. During the first 8 h post infection both strains multiplied and stimulated a similar immune response that involved expression of high levels of proinflammatory cytokines, the matrix metalloproteinase 9 (MMP-9), Interleukin-10, and the influx of white blood cells (WBC) into the

cerebro-spinal fluid (CSF). Virtually identical immune response was also elicited by intracisternal inoculation of either choline-containing or choline-free cell walls. At sampling times past 8 h strain D39Cho$^-$ continued to replicate accompanied by an intense inflammatory response and strong granulocytic pleiocytosis. Animals infected with D39Cho$^-$ died within 20 h and histopathology revealed brain damage in the cerebral cortex and hippocampus. In contrast, the initial immune response generated by the choline-free strain D39Cho$^-$licA64 began to decline after the first 8 h accompanied by elimination of the bacteria from the CSF in parallel with a strong WBC response peaking at 8 h after infection. All animals survived and there was no evidence for brain damage.

Using the same pair of strains in the murine sepsis model I demonstrated that this choline-associated virulence is independent of Toll-like receptor TLR-2 recognition. Also, despite the lack of virulence, choline-free strains of *S. pneumoniae* were able to activate splenic dendritic cells, induce production of proinflammatory cytokines as well as capsule-specific serum antibodies and develop protective immunity against subsequent challenge with the virulent strain. However, due to this transient engagement of the immune system the choline-free bacteria were rapidly cleared from the blood while the isogenic virulent strain D39Cho$^-$ continued to grow accompanied by prolonged expression of cytokines eventually killing the experimental animals.

From the meningitis and sepsis model I was able to conclude that choline allows bacteria to steadily grow within the host even in spite of an ongoing immune response. Therefore attachment of choline represents a mechanism to evade host clearance. In the last part of my thesis I was able to show that surface bound choline residues can confer resistance against the bactericidal activities of complement-free murine serum *ex vivo* and the cationic antimicrobial peptide Nisin *in vitro*. Removing or blocking the choline-residues with either choline-specific IgA TEPC-15 antibodies or human C-reactive protein abolishes the observed resistance. Passive application of these two immune molecules protects mice from pneumococcal colonization and sepsis, suggesting a possible mode of interaction between IgA antibodies / CRP and cationic antimicrobial peptides in their fight against bacteria, especially *S. pneumoniae*.

Zusammenfassung

Streptococcus pneumoniae ist ein gram-positives Bakterium und der Erreger von Lungen- sowie Mittelohrentzündung, Sepsis und Meningitis. *S. pneumoniae* ist umhüllt von einer dicken Zellwand, welche ein Charakteristikum von gram-positiven Bakterien darstellt. Diese Zellwand bildet zugleich die physische Oberfläche der Zelle und ist deshalb eines der Hauptziele des Wirtsimmunsystems. Das Ziel meiner Doktorarbeit war es zu verstehen inwiefern die Zellwandstruktur Auswirkungen auf die Virulenz des Erregers hat. Ich war vor allem an O-Acetyl-Seitengruppen und Cholinresten der bakteriellen Zellwand interessiert. Deshalb analysierte ich zwei Bakterienstämme, welche entweder vollkommen deacetyliert oder frei von Cholin waren.

Der erste Teil meiner Doktorarbeit beschäftigte sich mit einem vor Kurzem isolierten Bakterium, in welchem das Gen *adr* inaktiviert war. Es war bekannt, dass dieses Bakterium höchst Lysozym-sensitiv war. Von der DNA Sequenz des Gens und der biochemischen Analyse der Zellwand konnte ich darauf schliessen, dass jenes Gen *adr* für eine Peptidoglykan *O*-Acetyl-Transferase kodiert. Da dieses Enzym die *N*-Acetyl-Muraminsäure-Reste der Zellwand acetyliert, war diese Zellwandmodifikation in der entsprechenden *adr* Mutante nicht zu finden. Obwohl Acetylgruppen keinerlei Einfluss auf das Binden an humane Pharynxzellen *in vitro* hatten, zeigten *adr* Mutanten eine verminderte Fähigkeit den Nasopharynx von Mäusen zu besiedeln. Dies war auf die erhöhte Lysozym-Sensitivität zurückzuführen.

Im zweiten Teil meiner Doktorarbeit konzentrierte ich mich auf die Cholinreste der Zellwand. Diese Cholinreste sind Bestandteil der (Lipo)teichonsäuren der Zellwand. Cholin stellt eine Besonderheit in der Physiologie von *S. pneumoniae* dar, da das Bakterium auxotroph für diesen Aminoalkohol ist. Cholin muss also ein obligater und essentieller Bestandteil des entsprechenden Wachtumsmediums sein. Unser Labor war jedoch in der Lage eine *S. pneumoniae* Mutante (Cho^-) zu erstellen, welche fähig ist in cholinfreier Umgebung zu wachsen. Dank des Stammes Cho^- konnte ich die Auswirkungen von Cholin auf die Virulenz des Bakteriums während experimenteller Pneumokokken-Meningitis und Sepsis testen.

Für das Meningitis-Tiermodell wurden 11 Tage alten Wistar Ratten entweder der cholinhaltige Stamm D39Cho^- oder die cholinfreien D39Cho^-licA64 Bakterien in die Cisterna magna injiziert. Während den folgenden 8 h waren beide Bakterien in der Lage zu proliferieren und die Expression von proinflammatorischen Zytokinen, der

Matrixmetalloproteinase 9 (MMP-9), Interleukin-10 sowie das Einströmen von weissen Blutkörperchen zu stimulieren. Interessanterweise konnte eine vergleichbare Immunantwort auch durch die Applikation von aufgereinigten cholinhaltigen und cholinfreien Zellwänden ausgelöst werden. Nach der anfänglichen, achtstündigen Wachstumsphase waren nur die cholinhaltigen Bakterien in der Lage weiter zu wachsen. Dies führte zu einer stetigen Verstärkung der Immunantwort und dem Tod der Versuchstiere nach 20 h. Histopathologie der Gehirne zeigte massive Schädigung des zerebralen Cortexes sowie des Hippocampus. Im Gegensatz dazu sanken die bakteriellen Titer der cholinfreien Bakterien nach den ersten acht Stunden mit der Aktivierung des Immunsystems. Alle Tiere überlebten die Infektion unbeschadet.

Durch die Verwendung der gleichen Bakterien im intraperitonealen Maus-Sepsis Modell gelang es mir zu zeigen, dass die cholinassozierte Virulenz unabhänging von Toll-like Rezeptor 2 ist. Obwohl avirulent, waren die cholinfreien Bakterien dennoch in der Lage dendritische Zellen der Milz zu aktivieren und die Produktion von proinflammatorischen Zytokinen und kapselspezifischen Antikörpern zu induzieren. Diese vorrübergehende Aktivierung des Immunsystems war ausreichend um die cholinfreien Bakterien aus dem Blutkreislauf zu entfernen sowie eine protektive Immunität auszulösen, welche die Versuchstiere vor folgenden Belastungsinfektionen serotypspezifisch schützte. Im Gegensatz dazu wuchsen cholinhaltige Bakterien stetig, induzierten eine permante Expression proinflammatorischer Zytokine und führten zum Tod der Versuchstiere.

Von den Experimenten in den Tiermodellen konnte ich darauf schliessen, dass Cholin in der Zellwand für das bakterielle Wachstum in Gegenwart einer Immunantwort notwendig ist. Im letzten Teil meiner Doktorarbeit konnte ich zeigen, dass Cholinreste dem Bakterium die Fähigkeit verleihen in komplement-freiem Mausserum *ex vivo*, oder in Gegenwart des antimikrobiellen Peptids Nisin *in vitro* zu wachsen. Cholinfreie Bakterien sind dazu nicht in der Lage und werden von einem antimikrobiellen Bestandteil des Serums oder von Nisin beseitigt. Blockiert man die Cholinreste mit cholinspezifischen IgA TEPC-15 Antikörpern oder C-reaktivem Protein (CRP) kann diese Resistenz der cholinhaltigen Bakterien aufgehoben werden. Passive Applikation dieser beiden Moleküle kann sogar Versuchstiere vor Besiedlung des Nasopharynx sowie vor Sepsis schützen.

Table of Contents

1 Introduction

Streptococcus pneumoniae (“the pneumococcus”) is one of the first described human pathogens. Despite being known for more than a century all attempts to eradicate this important pathogen have been unsuccessful so far.

Since pneumococcal infections were commonly treated with antibiotics, studies on the pathology of this remarkable microorganism were neglected during the antibiotic era. Only the emergence of antibiotic resistant strains lead to a recurring interest in developing new vaccines that aim to eventually eliminate this bacterium.

To achieve this long term goal it is essential to foster knowledge of the molecular interactions between the human host and the pneumococcus. Since the majority of attacks launched by the immune system are targeted towards the bacterial surface, a grounding understanding of the bacterial cell envelope and human innate and adaptive immunity is crucial. Based on this information novel surface-associated virulence factors can be identified and used as potential vaccine candidates to dismantle the bacteria.

1.1 *Streptococcus pneumoniae*

1.1.1 History and Impact on Human health

S. pneumoniae is a gram-positive bacterium. It was originally isolated and recovered from rabbits infected with human saliva and described simultaneously by George Miller Sternberg and Louis Pasteur in 1881. Initially named *Diplococcus pneumoniae* the bacterium obtained its present-day’s name *Streptococcus pneumoniae* in 1974 referring to its phenotypical growth in either diplococcal shape or short filamentous chains.

S. pneumoniae played a pivotal role not only in the discovery of bacterial transformation but also in the designation of DNA being the carrier of the heritable information [1]. After showing that co-injection of an avirulent, but living rough strain with a heat-inactivated, non-viable extract of formerly virulent, encapsulated bacteria into mice resulted in the rapid killing of the animals Griffith concluded in 1928 that these rough bacteria must have been “transformed” within the host to acquire a virulence deter-

minant derived from the heat-inactivated cell extract [1]. Based on these findings Avery further fractionated the crude cell extracts and demonstrated that “nucleic acid of the desoxyribose type is the fundamental unit of the transforming principle”[1]. It was also in the naturally competent S*. pneumoniae* that “quorum sensing” between bacteria was described for the first time. The synchronized and transient induction of competence in these bacteria is dependent on the concentration of a secreted pneumococcal peptide in the growth medium [2].

Normally, *S. pneumoniae* is a commensal of the human nasopharynx. However, it can become an opportunistic pathogen and therefore is a major cause of mortality worldwide: a WHO report in 2007 estimated that over 1.6 million deaths per year are due to pneumococcal disease. Out of these worldwide fatalities, 0.7-1 million deaths can be attributed to the age group of children below 5 years old [3]. Pneumococcal infection is also one of the main contributors to the severity of viral respiratory disease. A retrospective study indicates that over 50% of the mortality of the 1918 influenza epidemic was caused by superinfections with *S. pneumoniae* [4]. Similarly, the fatality rate of Acquired immunodeficiency syndrome (AIDS) in sub-Saharan Africa is strongly linked to secondary pneumococcal infections [5].

Therefore *S. pneumoniae* impacts health systems in industrialized and developing countries as well, rendering this bacterium into one of the major global health concerns of the past and future.

1.1.2 Antibiotic Therapy and Vaccines

With community-acquired pneumonia being an important human disease the development of potent pneumococcal vaccines was always of highest priority. After initial vaccination trials using inactivated whole-cell preparations amongst miners in South Africa [6] it was the discovery that purified polysaccharides of the pneumococcal capsule are immunogenic and protective themselves [7], which lead to the development of polysaccharide vaccines in the 1940s, covering the capsular serotypes known at that time.

With the advent of antibiotics such as penicillin and sulfonamides, it was believed that these antimicrobials were the ultimate cures to pneumococcal infections and the im-

provement of existing vaccines was neglected. The first emergence of clinical multiple antibiotic-resistant *S. pneumoniae* in Johannesburg [8] diverted interest back to the development of advanced vaccines. Although vaccines against 23 of the major capsular types of pneumococci have been introduced in the 1980s [9], they are not effective in children, the primary targets of the pathogen.

For this reason, a heptavalent conjugate vaccine in which 7 important capsular polysaccharides are chemically linked to an immunogenic protein carrier has been developed and released just recently [6]. Extending the coverage presents increasing technical problems and the cost of the currently available conjugate vaccines is already prohibitive in the very countries where mortality of pneumococcal disease is the highest. In addition, pneumococcal serotypes responsible for most of pneumococcal diseases are known to be different between North versus South America, Asia, Africa and Europe.

However, all existing polysaccharide vaccines only comprise a few of the invasive serotypes, are not applicable for children or lead to the selection and emergence of non-vaccine serotypes [10]. Therefore infection with *S. pneumoniae* still ranks highest among all vaccine-preventable deaths and the development of alternative pneumococcal vaccines is essential. The design of such vaccines implies an elementary knowledge of the molecular processes involved in the pathology of this bacterial disease.

1.2 The Pneumococcal Surface

The bacterial surface plays a major role during pneumococcal disease. It represents the interface on which the prokaryotic cell interacts with antimicrobials and the host immune system. Therefore a profound understanding of its architecture and organization is critical to investigate the mechanisms underlying an infection with *S. pneumoniae*.

1.2.1 The Cell Wall

A common characteristic of gram-positive bacteria is the presence of a thick cell wall structure, which serves as the outer layer of these bacteria. This sturdy cell wall is mainly built of a gigantic macromolecule called peptidoglycan. Being the physical surface of the bacteria the cell wall serves as the anchor for capsular polysaccharides as well as for a variety of surface proteins. It maintains the integrity of the cell against the osmotic pressure and cellular turgor. Despite being the major determinant for cell shape the cell wall still has to retain a certain flexibility since it is involved in key physiological processes such as bacterial growth, cell division, autolysis and the trafficking of nutrients.

1.2.1.1 Peptidoglycan

In case of *S. pneumoniae* the basic structural components of the cell wall are long glycanstrains that consist of the alternating carbohydrates N-acetyl glucosamine (Glc*N*Ac) and N-acetyl muramic acid (Mur*N*Ac), the latter of which also serves as the attachment site for the so-called stempeptide, a pentapeptide consisting of L-alanine, D-isoglutamine, L-Lysine and two D-alanine residues (-L-Ala – D-iGln – L-Lys – D-Ala – D-Ala). After minor enzymatic modifications, donor and acceptor stempeptides of adjacent glycan strands can be crosslinked directly or via short, dipeptide bridges containing L-alanine or L-serin (L-Ala – L-Ala or L-Ala – L-Ser), thus forming the basic macromolecular scaffold of the cell wall, called “peptidoglycan” (see Figure 1).

The assembly of the peptidoglycan involves a variety of enzymes and can be divided into four major steps: a.) the intracellular synthesis of Lipid II, an undecaprenyl-

phosphate (UDP)-linked, membrane bound Glc*N*Ac – Mur*N*Ac – Pentapeptide precursor, b.) the transfer of Lipid II from the cytoplasm across the membrane onto the bacterial surface, c.) the extracellular extension of the nascent glycan strands with the sugar moieties of Lipid II by a transglycosylation reaction, followed by d.) a transpeptidation reaction that catalyzes the covalent linkage of stempeptides of close proximity between neighboring glycan strands ("crosslinking"). The transglycosylation and transpeptidation reactions are catalyzed by enzymes called penicillin-binding-proteins (PBPs) that are also the major targets of β-lactam antibiotics. Expression of low affinity derivatives of these PBPs confers penicillin resistance to the bacterium and can result in alternative cell wall structures.

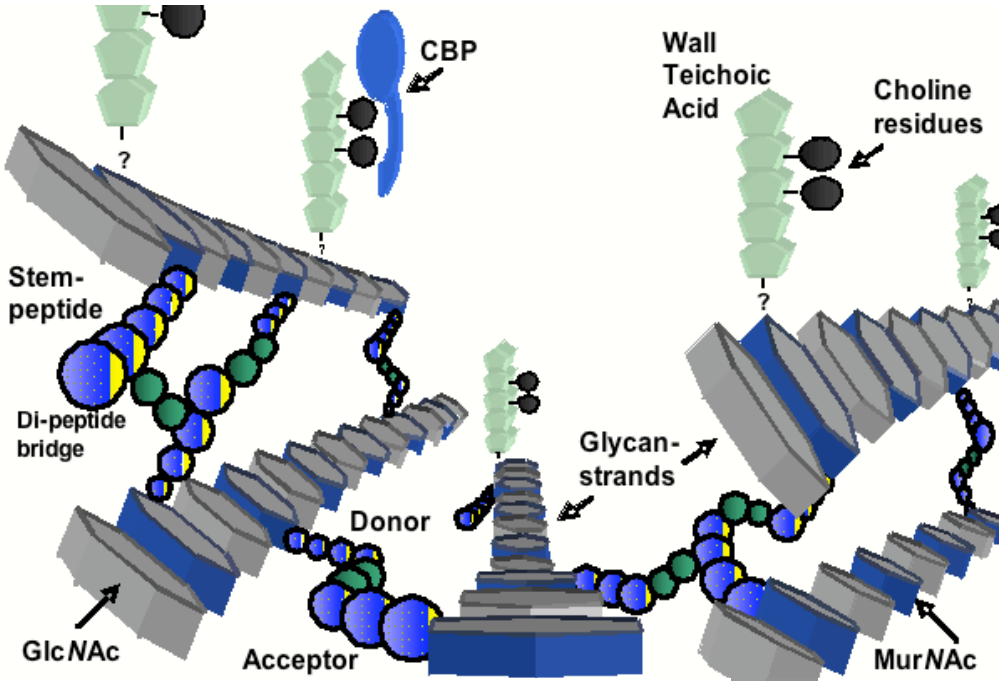

Figure 1: The pneumococcal cell wall.

Peptidoglycan strands consist of alternating N-acetyl-glucosamine (Glc*N*Ac) and N-acetyl-muramic acid (Mur*N*Ac) molecules. The peptidoglycan strands are linked to each other through stempeptides and short Di-peptide bridges. Wall teichoic acids (WTA) are attached to the Mur*N*Ac residues by an unknown linkage. Choline-binding proteins (CBP) bind to the choline residues of the WTA.

Generally, the composition and degree of crosslinking as well as other secondary modifications of the peptidoglycan backbone seem to be highly specific for different *S. pneumoniae* strains and their features. For instance, the penicillin-resistance of strain Pen6 was highly correlated to the preferential incorporation of the uncommon seryl-alanine dipeptide bridges, the synthesis of which is catalyzed by the gene prod-

ucts of alternative alleles in the *murMN* operon [11]. Also, the deacetylation of Glc*N*Ac components in strain R36A by a peptidoglycan-*N*-Acetyl-glucosamine-deacetylase (PgdA) enhanced resistance to lysozyme [12].

1.2.1.2 Capsule and Teichoic Acids

The peptidoglycan also functions as the anchor for two important families of carbohydrates of *S. pneumoniae*: the capsular polysaccharides and teichoic acids.

S. pneumoniae strains have a vast genetic repertoire for the production of the polysaccharide capsule which covers the outside surface of these bacteria with one or the other of 91 chemically different capsular polymers [13,14].These highly diverse, chemically distinct polysaccharides are the determinants of pneumococcal serotypes and are the foundation of the nomenclature of *S. pneumoniae.* Except for the serotype 3 capsule, all polysaccharides are covalently bound to the cell wall, although the exact site and nature of the bond remains elusive.

In spite of the great variety of capsular polysaccharides the genetic organization encoding for their synthesis shows a conserved and clustered pattern. Bordered by the flanking genes *dexB* and *aliA* the *cps* locus (capsular polysaccharide synthesis locus) of different pneumococcal serotypes contains genes common to all polysaccharides as well as unique and serotype specific gene sequences [15]. Genes encoding the biosynthesis of chemical components that can be shared with other metabolic processes of the cell (e.g. UDP-Glc*N*Ac) and that can be recruited from common cellular pools are located elsewhere on the bacterial chromosome [15]. Due to the conserved nature of the *cps* locus, capsular replacement by homologous recombination between two strains can occur and such capsular switching events have been frequently observed *in vivo* [16].

The second important group of surface polysaccharides, present in all pneumococcal serotypes, comprises the structurally related cell wall bound teichoic acid (WTA, formerly known as “C-polysaccharide”) and the membrane-bound lipoteichoic acid (LTA, formerly known as “Forssmann- / F-antigen”). Due to their characteristic attachment sites on the pneumococcal surface the –otherwise identical- two teichoic acid derivatives differ in one structural property: while WTA is covalently linked to the Mur*N*Ac

residues of the peptidoglycan via an unknown linker unit (see Figures 1,2), the incorporation of LTA into the bacterial membrane is achieved through a terminal glycolipid-anchor Monoglucosyldiacylglycerol (Glc-acyl$_2$Gro) (see Figure 2).

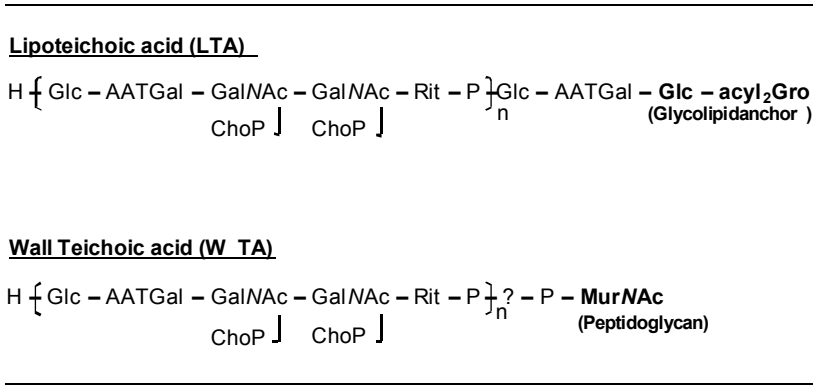

Figure 2: Structure of Teichoic Acids of S. pneumoniae.

The teichoic acid backbone common to both WTAs and LTAs consists of a basic, repeating subunit, composed of ribitol-5-phosphate (Rit-P), two D-N-acetyl-galactosamines (Gal*N*Ac), D-2-acetamido-4-amino-2,4,6-trideoxygalactose (AATGal) and D-glucose (Glc). It was also shown that teichoic acids can be subject to D-alanylation, most likely of the ribitol molecule [17]. A unique and characteristic feature of *S. pneumoniae* is the presence of the unusual amino-alcohol choline in its cell wall [18]. Depending on the strain, up to two phosphorylcholine molecules can be ester-linked to the two Gal*N*Ac residues of the teichoic acid backbone of WTA or LTA, respectively [19] (see Figure 2).

1.2.2 The Role of Surface-bound Choline in the Pneumococcal Physiology

S. pneumoniae is auxotroph for choline [20], making it an essential nutrient of the growth medium and *in vivo* environment. The bacterium incorporates choline into its cell wall [18] and attaches it to both the wall teichoic as well as the lipoteichoic acids, thus decorating its surface with this aminoalcohol. Choline serves as the anchor for a

family of choline-binding proteins (CBPs) on the pneumococcal surface, which play crucial roles in the bacterial physiology.

1.2.2.1 Synthesis of Cholinated Teichoic Acids

The biosynthesis of cholinated teichoic acids is a co-operative interplay of several parallel enzymatic reactions. While the teichoic acid precursor backbone is intracellularly assembled, choline has to be taken up from the extracellular environment, processed within the cell and bound to the teichoic acid precursor backbone, before the cholinated teichoic acid can be flipped across the membrane and connected to the peptidoglycan scaffold of the cell wall. In contrast to the fully understood choline metabolism the production of the teichoic acid backbone is only partially known. So far eight genes have been described to participate in the synthesis reactions. These genes are clustered in two genetic loci, designated *lic1* and *lic2* operon (see Figure 3).

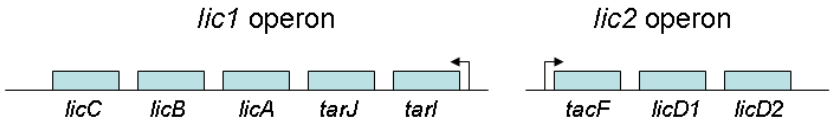

Figure 3: Genetic organization of the *lic1* and *lic2* operons.

Both operons are involved in the utilization of choline (genes *licA, licB, licC, licD1/2)* and the synthesis of teichoic acids (genes *tacF, tarI, tarJ*).

The only identified genes contributing to the production of the intracellular teichoic acid precursors (in particular the ribitol phosphate subunit) are located in the *lic1* operon [21]. TarJ, a NADPH-dependent alcohol dehydrogenase catalyzes the synthesis of ribitol 5-phosphate from ribulose 5-phosphate. Subsequently, the activation of ribitol 5-phosphate to cytidine 5'-diphosphate (CDP)-ribitol is achieved by the cytidylyltransferase TarI [21]. However, it is not known how CDP-ribitol is incorporated into the teichoic acid precursor molecule nor how the remaining TA backbone is synthesized.

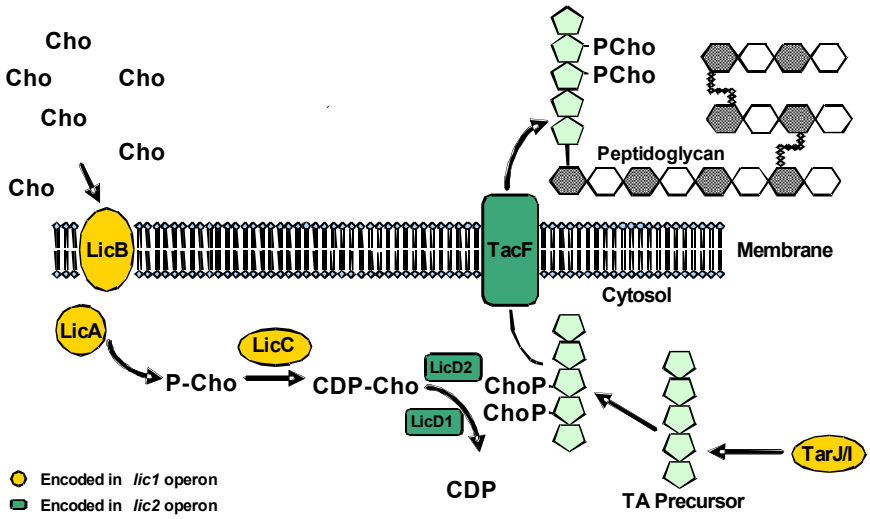

Figure 4: Synthesis of Cholinated Teichoic Acids (TA).

Extracellular choline is taken up by a choline transporter LicB, phosphorylated by an ATP-dependent choline kinase LicA, and activated by a phosphorylcholine cytidylyl transferase LicC. The phosphoryltransferases LicD1/D2 catalyze the attachment of choline to the TA acid precursor. Upon full cholination, the TA is transferred across the membrane by flippase TacF. The mechanism of attachment to the peptidoglycan scaffold is not known. Proteins TarJ/I are involved in the synthesis of the TA precursor.

In parallel, the acquisition of extracellular choline from the growth medium and its cytoplasmic processing has to take place. This is catalyzed by genes encoded in both the *lic1* and *lic2* operon. After choline uptake by a transporter LicB , and intracellular phosphorylation of the aminoalcohol by an ATP-dependent choline kinase LicA [22,23], the resulting phosphorylcholine is further activated to CDP-choline by the phosphorylcholine cytidylyl transferase LicC [22,24,25], in a CTP-dependent manner. The first three enzymes LicA/B/C are encoded in the *lic1* operon. Following these enzymatic reactions, gene products of the *lic2* operon catalyze the loading of teichoic acid chains with choline residues: each of the phosphorylcholine transferases LicD1 and LicD2 [26,27] attaches one CDP-choline derived phosphorylcholine residue to the two Gal*N*Ac residues of the TA precursors.

Once the cholination of the TA precursors is accomplished flippase TacF transports the choline-containing TAs onto the bacterial surface [28], where they are linked to the bacterial cell wall by a presently unknown mechanism (see Figure 4).

Teichoic acids were also described to be further modified: for instance the transfer of D-alanyl residues to the backbone is dependent on the presence of a functional *dlt* operon in the bacterial genome [17]. Interestingly, an extracellular phosphoryl-choline esterase Pce can secondarily remove choline-residues from TAs [29].

1.2.2.2 Choline-binding Proteins

Choline in the cell wall can dramatically influence physiological properties of *S. pneumoniae*. While the unique auxotrophic requirement for choline can be fulfilled by other structurally different amino-alcohols [30], the normal physiological properties of the bacterium require the trimethylamino group of choline. *S. pneumoniae* growing in media in which choline was replaced by ethanolamine show numerous abnormalities: they form long chains, do not autolyse and cannot undergo genetic transformation [31]. These fundamental phenotypical changes of the bacterium can mainly be attributed to the role of choline residue as an anchor for a versatile family of non-covalently bound choline-binding proteins (CBPs) (see Figure 1).

Besides their role in virulence (as discussed below) some CBPs contribute to the cell biology of the pneumococcus, especially to the physiology of the cell envelope. A common structural feature of CBPs is the existence of a C-terminal choline-binding domain [32]. Screening of available *S. pneumoniae* genomes for potential choline-binding domain sequences suggests the presence of 10 (in strains R6, D39) to 15 (in strain TIGR4) CBPs on the bacterial surface [33].

The first characterized and most intensively studied CBP is LytA, a *N*-acetyl-muramoyl-L-alanine-amidase, which is responsible for the phenomenon of autolysis. After reaching the stationary phase cultures of *S. pneumoniae* undergo self-induced lysis in a choline-dependent manner [31].

The physiological “purpose” of this process is not understood yet. The presence of LytA is also required for penicillin- and deoxycholate-induced lysis of the bacteria. Another CBP LytB, a β-*N*-acetylglucosamidase, cleaves the glycan strands and is responsible for daughter cell separation [34]. LytB mutants or bacteria grown in the presence of ethanolamine show impaired cell division and grow in long chains of cocci. LytC, a β-*N*-acetylmuramidase, is a lysozyme-like cell wall hydrolase [35], the

autolytic activity of which is regulated by the amino-terminal domain of another recently crystallized protein CpbF [36]. CBPs can also have regulatory effects on the transformation process of competent pneumococci as shown for CbpD, a putative murein hydrolase, which is supposed to be responsible for competence-induced cell lysis and DNA release [37].

Attachment of CBPs to the bacterial surface is dependent on the availability of vacant choline residues on the surface. A conceivable superior control mechanism on CBP attachment could therefore be the regulation of vacant choline-residues by another choline-binding protein Pce. Being a phosphoryl-choline esterase, Pce can remove phosphorylcholine residues from TAs [29].

1.2.2.3 Choline-independent Strains

Interestingly, the aforementioned ethanolamine induced phenotypical changes (e.g. chain growth, autolysis-deficiency) are exactly the same abnormalities shown by all known isolated choline-independent strains of *S. pneumoniae* when cultured in choline-free media [28,38,39] (see Figure 5).

Most of the studies on choline were performed on two choline-independent strains (Cho^-, JY2190), which were the only available and existing mutants for years. Just recently, several additional new mutants were obtained [28,40]. Most of these choline-independent strains were laboratory mutants isolated by an enrichment procedure in which the parental strain was serially passaged in a culture medium, the choline component of which was replaced by gradually decreasing concentrations of ethanolamine.

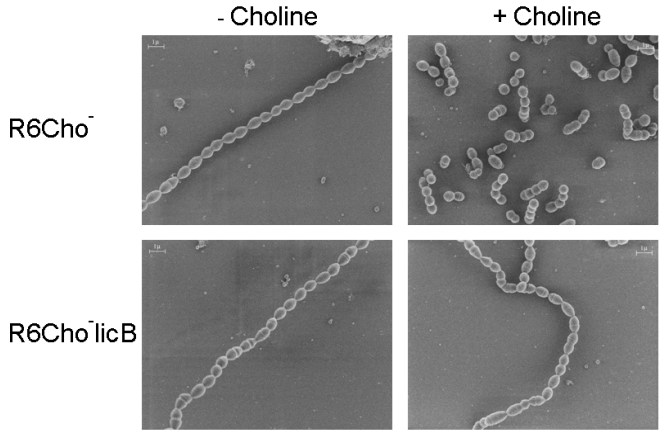

Figure 5: Morphology of R6Cho- and R6Cho-licB in choline-free and choline-containing media [41].

Both strains are able to grow in choline-free medium in long, autolysis-resistant chains. After supplying choline to the growth medium R6Cho- still has the ability to utilize choline and revert to the diplococcal wildtype phenotype. In contrast, mutants deficient in any of the choline utilization genes of the *lic1* operon (e.g *licB*) will maintain the choline-free phenotype even in the presence of exogenous choline.

This procedure eventually yielded mutants R6Chi [28], JY2190 [39] and a whole family of strains ranging from P023-P600 [40] which could grow in media completely lacking the aminoalcohol component. The first genetic and biochemical studies were performed on R6Chi and identified a single G→T point mutation in one of the genes of the *lic2* operon as the molecular basis of choline independence in this mutant [28]. During these studies, the gene *tacF* was discovered and proposed to encode for a polysaccharide transmembrane transferase ("flippase") that catalyzes transport of teichoic acid chains to the outer surface of the pneumococcal plasma membrane [28] (see Figure 6).

In contrast to the wildtype TacF that only transfers TA precursors with aminoalcohol moieties, it appears that the mutated form allows the transfer of unsubstituted precursors, thus enabling the survival of the bacteria (see Figure 6).

Analyzing strains JY2190 and P023-P600 revealed that differently located mutations in *tacF* can also confer choline-independence to the bacterium. So far neither the exact nature of the needed aminoacid alteration nor the required changes in structure and function of the TacF protein are fully understood.

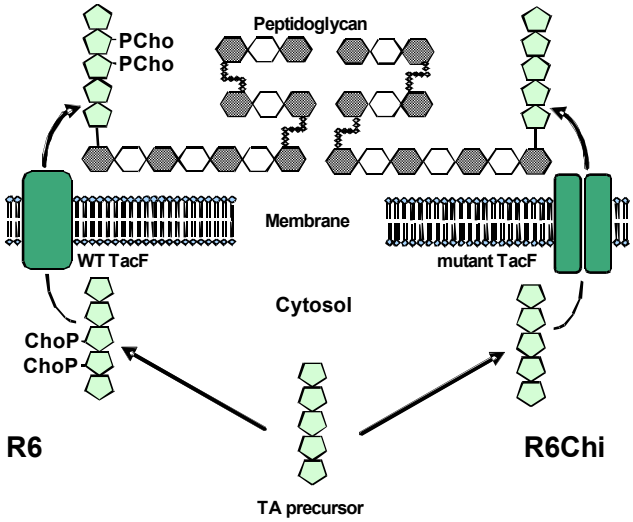

Figure 6: Mechanism of choline-independence in the Chi background.

In wildtype R6 the flippase TacF (left side of the panel) is restricted to transferring fully cholinated teichoic acid (TA) precursors to the surface, where they are attached to the cell wall. In contrast, flippase TacF of strain R6Chi (right side of the panel) possesses a mutation that also allows the transport of choline-free TA precursors across the membrane.

However, it seems that a modified TacF is the crucial element in delivering choline-independence to these strains.

The mechanism of choline-independence in the other extensively studied *S. pneumoniae* strain R6Cho$^-$ [38] was not known at the beginning of my thesis. R6Cho$^-$ shared many of the physiological properties of R6Chi / JY2190 / P023-P600 but had a more complex origin: it was isolated as the product of a heterologous genetic cross in which the recipient *S. pneumoniae* strain R6 was transformed with donor DNA from *S. oralis,* a streptococcal species that contains choline in its teichoic acid but has no auxotrophic requirement for it [38]. The resulting transformant R6Cho$^-$ lost its auxotrophic need for choline, and was the bacterial strain predominantly studied in this work.

All choline-independent strains will maintain the wildtype phenotype whenever choline is available. Choline utilization can be avoided by deletion of any of the genes in the lic1 operon (e.g. *licA*, *licB*, *licC*) (see Figure 5).

1.2.3 Surface Proteins of *S. pneumoniae*

The pneumococcus has numerous surface proteins that can be divided into four major groups, depending on their secretion pathways and the nature of the chemical bond linking them to the surface.

The first group comprises proteins that are covalently attached to the peptidoglycan. These proteins contain a common c-terminal LPxTG-motif, which is recognized by Sortase A (SrtA), a transpeptidase that catalyzes the covalent linkage of these proteins to the peptidoglycan [42]. It was shown that the sortase-dependent proteins neuraminidase A (NanA) and β-galactosidase (β-Gal) were released into the growth medium by *srtA* mutants of the R6/D39 background. Additional sortase genes (*srtB*, *srtC*, *srtD*) were found in strain TIGR4 associated with the strain's virulence [42].

A second group of surface proteins - only found in pneumococci in such abundance – are the aforementioned choline-binding proteins (CBPs), named after their non-covalent binding to the unusual choline-residue of the cell wall (see above).

Lipoproteins of *S. pneumoniae* include proteins that function in mechanisms as diverse a manganese-transport and adhesion (PsaA, pneumococcal surface adhesion A), iron-uptake (PiaA and PiuA) or as chaperons belonging to the peptidyl-prolyl isomerase family (PPIases) [43].

Additional proteins that cannot be assigned to any of the three above mentioned, classical groups are summarized as "moonlighting" proteins [43], but will not be discussed in further detail here.

1.3 *Streptococcus pneumoniae* and the Host Immune System

In the majority of cases *S. pneumoniae* primarily colonizes the mucosal surface of the nasopharynx in a transient and asymptomatic manner. However, for unknown reasons, bacteria can occasionally descend to the lungs and induce potentially lethal pneumonia. A dysfunctional Eustachian tube can also grant access to the middle ear, leading to otitis media [44]. Most likely originating from the lungs, the bacteria can disseminate into the bloodstream and lead to systemic bacteraemia or even lethal septicemia. The most severe outcome of pneumococcal disease is the infection of the brain and meningitis.

Mucosa, bloodstream and brain are immunologically very different host compartments. In all these *in vivo* environments *S. pneumoniae* has to defend itself against versatile immune effectors. To ensure survival, the bacterium is equipped with an arsenal of virulence factors to react and adjust to these host defense strategies.

The following chapter will describe the major modes of infection and focus on selected “host-pneumococcus-interactions”, particularly important to understand the data presented in this work.

1.3.1 Immune response to *S. pneumoniae*

Upon infection of the human host *S. pneumoniae* is the target of a variety of defense mechanisms. The immediate host measures to pneumococcal infection involve pathogen-unspecific immune effector mechanisms of the innate immunity that seek to eradicate the bacteria.

The simultaneous induction of inflammation helps to amplify this immune response. Inflammation also jumpstarts the host’s adaptive immunity that will eventually kill the invaders and lead to a (long-term) pathogen-specific immunological memory.

1.3.1.1 Innate Immunity

Pneumococci trying to infect the human host have to successfully overcome innate immunity, which constitutes the first, intrinsic line of defense. The germ-line encoded

innate immune effector mechanisms found on the mucosa are different from the one's present in the bloodstream. Nevertheless, they are always directed against conserved molecular structures that are abundant in a multitude of pathogens.

For example, an effective defense can already be achieved by physical barriers. Cells of mucosal epithelia are sealed by tight junctions and secrete viscous mucus to avoid colonization and invasion. The mucus covers and agglutinates invading bacteria which will subsequently be expelled by the movement of the cilia. The existing commensal microflora of the target tissues competes with intruding pathogens for nutrients and physical space inhibiting their attachment. Another very effective mechanism to avoid bacterial proliferation is to establish a pH barrier or to shift the temperature away from the bacterium's growth optimum, which occurs during inflammation or fever [45].

Besides these straightforward but simple defense strategies, innate immunity also possesses manifold sophisticated molecular strategies to tackle bacteria.

For instance, lysozyme can be found in saliva and mucus and is especially effective in the clearance of gram-positive bacteria, as it directly attacks the cell wall and the stability of the whole bacterial cell itself. Being an N-acetylmuramide glycanhydrolase it cleaves the β(1→4) glycosidic bond between the Glc*N*Ac and Mur*N*Ac residues of the peptidoglycan, resulting in the enzymatic degradation of the cell wall and the lysis of bacteria [46].

Another way to fight bacteria is the secretion of cationic antimicrobial peptides (CAMPs). Studies in human patients as well as in animal models demonstrated elevated levels of these peptides during bacterial mucosal colonization, septicemia and meningitis [47,48,49,50,51]. It is believed that the peptides' mode of action is mainly mediated through their cationic charge, which attracts them towards the anionic phospholipids of bacterial membranes. Due to their hydrophobic nature the peptides are able to disrupt the membrane and eventually kill the pathogen [52].

Also found on mucosal surfaces is the secretory, dimeric immunoglobulin A (IgA). IgA is produced in high abundance, and is assumed to play a crucial role in the protection of the host against mucosal pathogens [53]. However, the exact effector functions of this immunoglobulin are not fully understood. It is speculated that IgA blocks the ad-

herence of bacteria to mucosal epithelia or neutralizes bacterial toxins [45]. A monomeric form of IgA is found in the serum. Since IgA is only a weak inducer of complement (see below) it is concluded that serum IgA has to have additional, unknown microbicidal effects [53]. Interestingly, naturally occurring choline-specific serum IgG and IgM antibodies were shown to be present in murine and human serum and can be protective against pneumococcal infection [54].

Once in the tissues or the bloodstream, bacteria have to face additional powerful defense mechanisms.

A very well characterized effector is “complement”. The major goals of this complicated system are to directly kill pathogens, to coat (“opsonize”) the microbial surface and to induce inflammation at the site of infection (see below). In general, opsonization with immune molecules (e.g. complement, antibodies…) facilitates the ingestion of pathogens by macrophages and neutrophils. The activation of complement can be initiated through the recognition of bacteria by complement complex C1q in combination with IgG and IgM (“classical pathway”), by Mannose-binding lectin (MBL) that preferably recognizes mannose or Glc*N*AC (“lectin pathway”) [55,56] or via spontaneous binding of complement on the pathogen’s surface (“alternative pathway”) [45]. In contrast to pathogens, host cells are protected against the spontaneous deposition of complement proteins induced by the alternative pathway due to presence of certain surface proteins, such as factor H, which displaces erroneously bound complement factors. Despite the differences in activation, all three pathways merge at the formation of a C3 convertase that converts the serum factor C3 into the inflammatory C3a molecule and C3b, the latter of which eventually opsonizes the whole pathogen. Serum factors C5b-C9 form the so-called membrane-attack complex (MAC) and lead to membrane perforation and subsequent killing of bacteria. However, due to its thick peptidoglycan layer *S. pneumoniae* is protected against the microbicidal effects of the MAC [57].

Another immune molecule contributing to the clearance of *S. pneumoniae* is the C-reactive protein (CRP). It is a liver-synthesized acute phase serum protein, rapidly secreted during inflammation. Although it was originally discovered in the serum of patients suffering from pneumococcal pneumonia [58], later studies also demonstrated its presence in the upper respiratory tract and in nasal secretions [59]. C-

reactive protein was named after its affinity for WTA (formerly C-polysaccharide). Further investigations revealed that CRP has a high specificity towards the phosphorylcholine residue of either WTA or LTA of *S. pneumoniae* [60]. Just recently another choline-specific pentraxin Serum amyloid P (SAP) was described [61]. Using direct competition assays it was determined that the overall avidity of SAP towards the choline residue was lower than the one of CRP [61]. Both, CRP and SAP can opsonize and activate complement via the classical pathway as well as induce phagocytosis by macrophages and neutrophils [62,63,64]. It was shown that the presence of SAP or heterologous human CRP is important to control pneumonia and bloodstream infections of *S. pneumoniae* in mice [62,65].

Innate immunity also possesses several cellular defense mechanisms. After crossing the epithelial barrier and infecting submucosal tissues, *S. pneumoniae* will encounter resident phagocytic host cells, called macrophages. In the course of an infection more phagocytic white blood cells, the neutrophils, will evade from the bloodstream and influx into the site of inflammation. These phagocytes express numerous surface receptors which recognize bacterial components or previously opsonized pathogens and trigger the engulfment and ingestion of the microorganisms. The macrophage mannose receptor was shown to bind to pneumococcal capsular polysaccharides, although the exact epitope on the bacterial polymers could not be determined yet [66]. Scavenger receptors, which represent another receptor family, target negatively charged LTA or peptidoglycan components [67]. In particular the macrophage receptor with collagenous structure (MARCO) was shown to play a crucial role in the clearance of *S. pneumoniae* by alveolar macrophages during pneumonia [68]. Similarly, the removal of pneumococci by macrophages of the marginal zone of the spleen depends on the presence of a C-type lectin receptor named SIGN-R1 [69]. Complement and Fc receptors facilitate the uptake of opsonized microorganisms through the recognition of bound complement factors or antibodies, respectively.

Upon internalization bacteria are contained in a vesicular phagosome, which - upon fusion with lysosomes - becomes a phagolysosome. Within this compartment bacteria are exposed to a whole array of microbicidal mechanisms, ranging from acidification, enzymatic degradation or nutrient deprivation to cationic antimicrobial peptides.

A recently discovered altruistic defense mechanism of neutrophils is the expulsion of neutrophil extracellular traps (NETs). Hereby, neutrophils eject DNA which forms a web of chromatin that traps and kills bacteria together with other released bactericidal agents [70,71].

1.3.1.2 Induction of Inflammation

In parallel to the innate immune system's effort to eradicate attacking pathogens immediately, the host initiates an inflammation which will orchestrate a complex interplay of several immune mechanisms in order to amplify the overall immune response.

Dendritic cells (DCs) and macrophages are the major modulators of inflammation. Expressing pattern recognition receptors (PRRs) they permanently sense their surrounding environment and the phagocytosed materials for the presence of pathogens by scanning for highly conserved pathogen-associated molecular patterns (PAMPS). For instance, the family of Toll-like receptors (TLRs) comprises ten members, out of which TLR-2 binds to pneumococcal LTA, TLR-4 to the major pneumococcal toxin pneumolysin (see below) [72] and TLR-9 to unmethylated CpG motifs specific to bacterial DNA [73]. The intracellular protein Nucleotide-oligomerization domain 2 (Nod 2) receptor detects cytosolic muramyl-dipeptides of *S. pneumoniae*, a cell wall component consisting of muramic acid and L-Ala and D-iGln residues [74]. Two members of a recently discovered family of Peptidoglycan-recognition-proteins (PGRPs), the amidase PRGP-L [75] and PGRP-S, were shown to contribute to *S. pneumonia* recognition and clearance although the exact mechanism could not be clarified yet [76].

Upon detection of intruding pathogens, DCs are activated and start to secrete proinflammatory cytokines, called Interleukin 1β (IL-1β), IL-6, Tumor Necrosis Factor alpha (TNF-α), CXCL8 (formerly IL-8) and IL-12. Especially IL-1β, IL-6 and TNF-α mediate local inflammation characterized by the constriction of adjacent blood vessels, the activation/permeation of the vessel's endothelia and the detour of the blood/lymph flow to the infection site. A similar local inflammatory response can also be triggered by small molecules of the activated complement cascade such as C3a, C4a and C5a. This inflammation process alleviates the influx of innate effector molecules (complement, antibodies) or macrophages and neutrophils into the site of inflammation.

The consecutive interaction of adherence molecules on both, the activated vascular endothelium (e.g. Selectins, ICAM-1) and circulating, activated neutrophils (e.g. Integrins), leads to a first reversible “rolling” of neutrophils on the endothelium followed by the tight binding to each other. Attached neutrophils cross the basement membrane and migrate within the tissue along the chemical gradient of chemokine CXCL8 towards the infectious focus, where they can execute their bactericidal effects.

To assure the constant supply of reinforcing immune cells IL-1β, IL-6 and TNF-α also have systemic effects and stimulate the production of neutrophils in the bone marrow. These cytokines also regulate the body temperature and lead to fever, which aims to inhibit the pathogens’ growth. They also induce the acute phase response with the production of CRP, SAP [62] and MBL in the liver. These molecules can trigger complement and phagocytosis by neutrophils. CRP was also shown to elicit IL-1β and TNF-α secretion *in vitro* [58].

Another effect of TNF-α is to stimulate the drainage of dendritic cells within the lymph fluid flow towards the lymph nodes where DCs mature and initiate an adaptive immune response [45].

1.3.1.3 Adaptive Immunity

Immature dendritic cells aim to detect pathogens with PRRs. Then they enzymatically disassemble proteins of the intruder and present the digested pathogen-derived peptide components on surface molecules named major histocompatibility complex I or II (MHC-I, MHC-II). Simultaneously the DC secretes cytokines (see above), stops phagocytosis and MHC turnover. Thus it “freezes” its surface and presents these short pathogen-derived peptide fragments. Activated DCs also upregulate costimulatory molecules CD80 and CD86 and start migrating to the lymph nodes [45].

In the lymph node they get in close proximity to naïve T-cells. The T-cell receptor of each T-cell clone has a certain specificity and can bind to surface MHC molecules on DCs. Once a T-cell specifically recognizes a pathogen-peptide-MHC complex, and additionally receives a second signal from the costimulatory molecules CD80 or CD86 on DCs, this T-cell clone will be activated and starts to proliferate.

In the case of a protein-specific adaptive immune response against the extracellular pathogen *S. pneumoniae* the differentiated effector T-cells activate antigen-specific B-cells. These B-cells become antibody-secreting plasma-cells that can potentially establish a long-term immunological memory. The peptide-specific antibodies aim to opsonize bacteria, stimulate complement deposition or interfere with the pathogen's attachment to target tissues. Antibodies to pneumococcal proteins PsaA, CbpA and PspA were shown to be protective against nasopharyngeal colonization [77,78,79].

On the other hand, production of naturally occurring antibodies specific to the capsular polysaccharide and phopshorylcholine components of *S. pneumoniae* appears to be T-cell-independent (TI). A certain subpopulation, called B-1 cells, can directly be activated upon contact with these pneumococcal TI antigens and contributes to the rapid secretion of circulating antibodies of the IgM or IgG isotypes. However, a T-cell independent immune response does not lead to a long term immunological memory [80].

These two described immune mechanisms are also the crux of the efficacy of pneumococcal vaccines. A protein specific vaccine would be able to induce long-term immunity in the host. Yet, no such immunogenic pneumococcal protein compound common to all strains and serotypes has been identified that satisfactorily induces immunity. In contrast the known polysaccharide vaccines consist of TI antigens and do not establish strong and powerful long-term protection. A recently released polysaccharide vaccine Prevnar tries to overcome these problems by chemically coupling the polysaccharide to the cholera toxin protein thus triggering an immunological memory. Unfortunately, the serotype coverage of this vaccine is limited to the restricted amount of polysaccharide molecules that can be attached to the protein carrier molecule.

1.3.2 Virulence Factors in Pneumococcal Disease

S. pneumoniae is very flexible and infects various host tissues and organs. To adapt to these various growth environments, pneumococci can regulate the expression of their capsule, a process named "phase variation" [81] (see Figure 7). Similarly, the bacteria switch between two growth modalities, planktonic life in the bloodstream and

biofilm growth on lung tissues, in the middle ear (otitis media) and during meningitis [82]. Both - the two phases as well as the two growth modalities - are characterized by specific gene expression profiles including multiple virulence genes.

1.3.2.1 Mechanism of Colonization

Pneumococcal disease starts with the colonization of the host. This process can be divided into two sequential components: the bacterium has to evade innate immunity of the mucosa first, before multiple receptor-ligand interactions promote adherence to nasopharyngeal and lung epithelial tissues.

A major virulence factor of *S. pneumoniae* is the pneumococcal capsule, which fulfills specific tasks in respect to the site of infection. Its role in the mucosal compartment is to reduce the agglutination of pneumococci by mucus and to limit the bacterium's ejection from the host system [83]. To inhibit the hydrolytic and bactericidal effects of the abundantly present lysozyme the bacterium expresses PgdA, a protein that enzymatically N-deacetylates the Glc*N*Ac units of the peptidoglycan strands and confers resistance [12]. *S. pneumoniae* has also to compete with the residential, commensal microflora of these organs to create its own growth niche. Interestingly, it was demonstrated that the production of hydrogen peroxide by *S. pneumoniae* efficiently displaces *Staphylococcus aureus* from the nasopharynx [84] allowing *S. pneumoniae* to settle. To repel secreted cationic antimicrobial peptides from its membrane the pneumococcus attaches positively charged D-alanyl residues to its teichoic acids, thus lowering not only the net negative charge of the surface but also its susceptibility towards CAMPs [17].

After bypassing the immune system *S. pneumoniae* possesses several molecular adhesion strategies, with which the bacterium is able to colonize host epithelia.

Pneumococcal phosphorylcholine residues show structural homology to the host molecule Platelet-activating factor (Paf), thus promoting binding to its respective receptor (rPaf) on the epithelial cells of the mucosa [85]. The pneumococcus even uses the host cell's recycling pathway of the rPaf to cross the epithelial barrier and enter the host system [85]. Interestingly, the surface decoration with phosphorylcholine residues is a common trait featured by various pathogens that colonize the respira-

tory system [32]. Choline-binding protein A (CbpA) also functions as an adhesion molecule of pneumococci recognizing polymeric Ig receptors on host cells [43].

Besides choline-associated binding mechanisms *S. pneumoniae* has the ability to interact with glycoconjugates on host cells. For example, the addition of N-acetylglucosamine–β-1-3-galactose (Glc*N*Acβ1→3Galβ) inhibits pneumococcal binding to human pharyngeal cells *in vitro* [86]. Similarly, other oligosaccharides were described that promote attachment of pneumococci to epithelial cells [87,88,89].

The recently discovered pneumococcal pilus was also suggested to contribute to host cell adherence and virulence of pneumococci [90]. However it does not seem to be a major adhesin of *S. pneumoniae* since screening of different pneumococcal serotypes revealed that the pilus genes are present only in the minority of pneumococcal strains [91].

Although it has protective effect against the mucus, the polysaccharide capsule of the bacteria represents a major problem for the adherence process, since it masks and shields the underlying cell wall localized adhesion molecules needed for attachment to epithelia. Therefore the surface of the pneumococcus varies between two different growth phases, a “transparent”, colonizing one, in which capsular polysaccharide content is the lowest and expression of cholinated teichoic acids and adhesion molecules (e.g. CbpA) is high, and an “opaque”, invasive phenotype, in which the bacterium is protected by a thick capsule against defense mechanisms occurring upon invasion [81] (see Figure 7). It also appears that the capsule interferes with the formation of biofilms, the preferred growth modality of *S. pneumoniae* during colonization [82,92]. Actually, the common upregulation of some genes (e.g. *nanA*) found in expression profiles of transparent, colonizing colonies and biofilm-derived cells might suggest a correlation between these two modalities of growth [82].

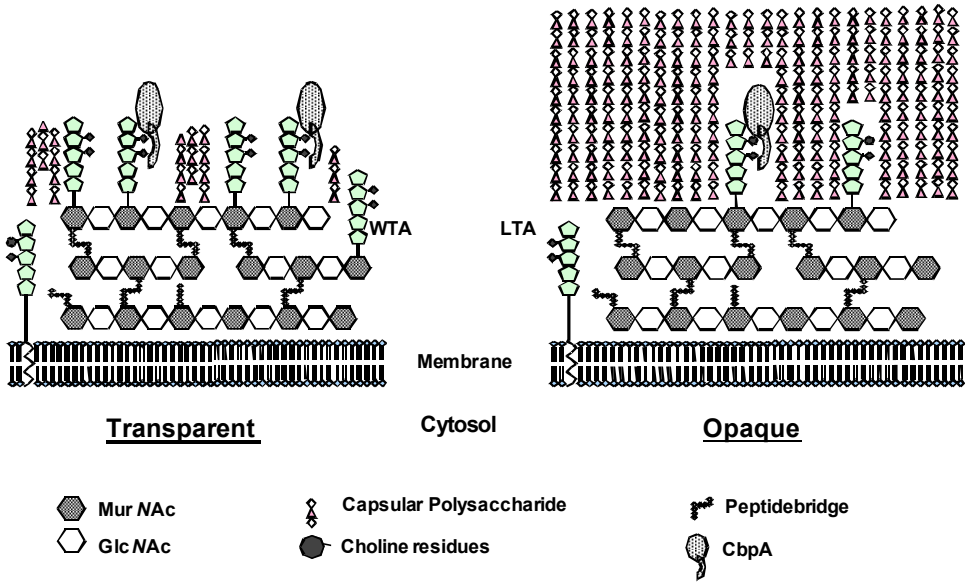

Figure 7: Phase variation of S. pneumoniae [81].

Phase variation is dependent on the site of infection. Pneumococci show transparent morphology during colonization, in which capsular polysaccharide expression is repressed and upregulated adhesive structures such as Choline-binding protein A (CbpA) and cholinated teichoic acids are exposed. During bloodstream growth *S. pneumoniae* has an opaque appearance, as a result of enhanced capsule expression, protective against immune clearance mechanisms such as complement. WTA: Wall teichoic acid, LTA: Lipoteichoic acid.

1.3.2.2 Mechanisms of Invasive disease

To establish an invasive disease like bacteraemia or meningitis, *S. pneumoniae* faces certain obstacles such as the lung epithelia or blood-brain barrier that the bacterium has to pass. To achieve this it either takes advantage of already existing host cell pathways or actively perforates these tissues by secreting bacterial toxins or induction of inflammation. Change from one infection site to another is also accompanied by pneumococcal phase variation and altering growth modalities of the bacteria.

As already mentioned above, the choline-dependent rPaf recycling pathway is probably the most elegant way of pneumococci to traverse through host cells and cross these physical barriers [85]. Another strategy of the bacteria is a more drastic approach. During lung colonization expression of the pneumococcal autolysin LytA production is maximal. Dependent on choline residues in the cell wall and LytA, pneumococci undergo self-induced autolysis during stationary phase *in vitro* [31]. *In vivo*, LytA might act as a direct virulence factor and trigger the release of cell wall

compounds. The induced inflammation may induce permeability of host barriers. The application of cell wall fragments into various animal disease models demonstrated inflammatory activity of these molecules [76]. Autolysin LytA can also have an indirect impact on virulence by releasing an intracellular virulence factor: the major pneumococcal toxin pneumolysin. Pneumolysin is a pore-forming toxin that attacks cholesterol-containing membranes. It induces lysis and apoptosis in host cells, such as neutrophils [93] and macrophages [94]. It also has inflammatory potential as it is recognized by TLR-4 [72] and complement. By destruction of whole tissues such as lung epithelia [95,96] or the blood-brain barrier [97] this toxin might play a crucial role in the invasiveness of pneumococci. It is also responsible for brain damage during meningitis [98]. However, the interplay between LytA and pneumolysin is still controversial and a matter of speculation [99,100,101].

Having invaded the bloodstream, the pneumococcus has to cope with additional immune effector mechanisms. In the blood the bacteria exist in a planktonic life style [82] characterized by the production of a bigger capsule. The major protective effect of the capsule is directed against the complement system. It was shown that the pneumococcal capsule inhibits the deposition of C3b on the bacterial surface [102]. Additionally *S. pneumoniae* produces a surface protein called Factor H-binding inhibitor of complement (Hic) that recruits and attaches the host's complement regulator Factor H (see above) to the pneumococcal surface thus preventing the binding of C3b as well [103]. These two virulence mechanisms of pneumococci abolish complement-mediated opsonophagocytosis of the bacteria.

The host also reacts to the secretion of pneumolysin since platelets can sense this toxin in a TLR-4 dependent manner and stimulate neutrophils to produce NETs. NETs are present in the vascular system of the lung and in the liver during sepsis. Since NETs consist of chromatin, *S. pneumoniae* expresses a surface bound endonuclease EndA that degrades DNA.

1.4 Aim of the work

The cell wall of *S. pneumoniae* is the outer surface of this gram-positive bacterium. During an infection it is exposed to the host's immune system and represents the ma-

jor target. The aim of my thesis was to understand the contribution of acetyl-groups and choline residues of the cell wall to the pathology of pneumococcal disease. Therefore I analyzed two pneumococcal mutants (one free of acetyl-groups and the other one lacking the choline residues) not only biochemically *in vitro* but also in respect to their virulence in various animal models of pneumococcal disease. The work on the choline residues was especially intriguing since *S. pneumoniae* has an auxotrophic requirement for this nutrient. Therefore, the recent construction of a choline-independent strain Cho^- allowed me for the first time to investigate the role that this aminoalcohol plays in the virulence of the pneumococcus.

2 Results

2.1 O-Acetylation of Peptidoglycan

„Crisostomo MI, Vollmer W, Kharat AS, Inhülsen S, ***Gehre F****, Buckenmaier S, Tomasz A. Attenuation of penicillin resistance in a peptidoglycan O-acetyl transferase mutant of* Streptococcus pneumoniae*. Mol Microbiol. 2006 Sep; 61(6):1497-509.“*

Chapter Summary:

My first studies on the effect of cell wall structure on pneumococcal virulence involved the recently discovered O-acetyl groups of the peptidoglycan. Previous work using mariner mutagenesis in search of additional genetic determinants that may further attenuate the level of penicillin resistance identified gene adr (for attenuator of drug resistance) in strain Pen6 [104]. DNA sequence similarity and HPLC analysis of cell walls suggested that the pneumococcal adr gene may be related to the peptidoglycan O-acetyltransferase of S. aureus [105]. This was also consistent with the increased sensitivity of the pneumococcal adr mutant to lysozyme [104] and its loss of colonizing capacity of the murine nasopharynx.

2.1.1 Identification of Adr as an O-Acetyltransferase using HPLC

To directly test this proposition cell walls were prepared from Pen6 and Pen6*adr* under conditions that allow retention of the *O*-acetyl groups in the peptidoglycan [105].

Figure 8A shows the HPLC elution profiles of cell wall muropeptides. In contrast to the identical stem peptide patterns, the muropeptide elution profile showed clear differences between Pen6 and its *adr* mutant. The extra peaks present in the parental strain would disappear upon treatment with mild alkali, i.e. under conditions that are known to remove *O*-acetyl groups. The HPLC profiles of the NaOH treated Pen6 peptidoglycan and the untreated peptidoglycan of the *adr* mutant were indistinguishable.

Alkaline-labile muropeptides were also present in the cell wall of the strain R6 (Figure 8B).

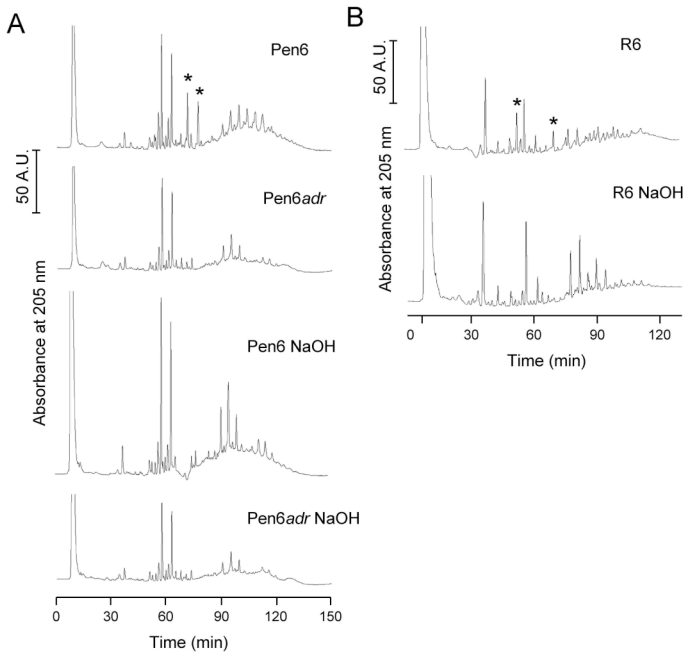

Figure 8: HPLC profiles of muropeptides from cell walls of Pen6, Pen6*adr* and R6 prepared under conditions that preserve *O*-linked acetyl groups. (A)

Muropetides of Pen6 and Pen6*adr*. Pen6 contains muropeptides with an *O*-acetyl group (asterisk) that are missing in Pen6*adr* and that are removed by treatment with sodium hydroxide. **(B)** Muropeptides of R6. The alkaline-sensitive muropeptides containing an *O*-acetyl are labelled with an asterisk.

Six of the major peaks present in the monomeric region of the chromatogram of strain Pen6 were isolated, desalted and analyzed by mass spectrometry (see Figure 9A). The determined molecular masses of the two major monomeric muropeptides and their *O*-acetylated variants were in accordance with the calculated molecular masses (Table 1). Partial fragmentation during mass spectrometrical analysis proved the presence of an unmodified *N*-acetylglucosamine residue in the *O*-acetylated muropeptides (data not shown).

This is expected, because *O*-acetylation occurs at the *N*-acetylmuramic acid residues in other species. In addition, the molecular mass of a *N*-deacetylated variant of one monomer was obtained, the partial fragmentation of which resulted in the release of a

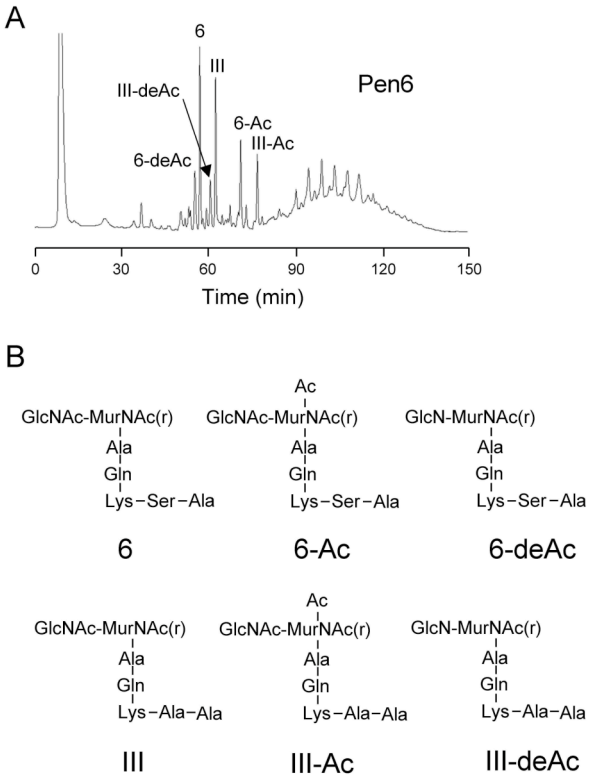

Figure 9: HPLC profile of muropeptides from Pen6.

(A) HPLC chromatogram with the major monomeric muropeptides. **(B)** Proposed structures of the unmodified (6 and III), *O*-acetylated *N*-acetylmuramic acid (MurNAc-O-acetylated) (6-Ac and III-Ac) and *N*-deacetylated *N*-acetylglucosamine (GlcNAc-N-deacetylated) (6-deAc and III-deAc) muropeptides.

Table 1: Molecular masses of muropeptides

muropeptide[1]	**mass (H^+-form) [amu]**	**calculated mass (H^+-form) [amu]**
6	984.4	984.5
6-Ac	1026.7	1026.5
6-deAc	n.d.[2]	942.5
III	968.8	968.5
III-Ac	1010.9	1010.5
III-deAc	926.4	926.5

1) see Figure 9
2) no data obtained

glucosamine residue. The proposed structures of the major monomeric muropeptides from Pen6 are shown in Figure 9B.

As further proof for the presence of *O*-acetyl groups, the cell walls of Pen6 and the *adr* mutant were treated with sodium hydroxide and the release of acetate was tested. Acetate released from strains Pen6 and R6 was identified by ion-exchange chromatography. No acetate release was detectable in Pen6*adr* (see Figure 10).

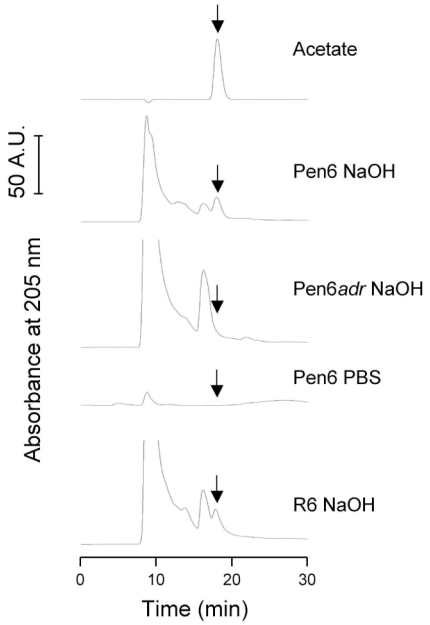

Figure 10: Detection of the release of alkaline-labile acetate from the cell walls by ion exchange chromatography.

Cell walls of strains Pen6, R6, and Pen6*adr* were treated under alkaline conditions and the released acetate was detected by ion exchange chromatography. There was no acetate release from Pen6 with phosphate-buffered saline (PBS). Arrows indicate the retention times of acetate.

2.1.2 Impact of O-Acetylation on Nasopharyngeal Colonization

Since gene *adr* is an O-acetylase that confers resistance towards lysozyme it was interesting to test whether respective mutants show impaired capacity to colonize the nasopharynx, a mucosal host compartment in which lysozyme is abundantly present as an innate effector molecule. Two different parameters can also play an important role during colonization which have to be excluded. On the one hand it is conceivable that the *O*-acetyl group can directly bind to nasopharyngeal epithelial cells and func-

tion as an adhesive molecular structure. On the other hand a slower growth rate of the mutant bacteria can limit the ability to colonize.

2.1.2.1 Adherence and Invasion of Pen6/Pen6*adr* to the Pharyngeal Cell Line Detroit 562

To understand the role of the *O*-acetyl group in adhesion and invasion, 10^6 CFU/ml of either Pen6 or Pen6*adr* were incubated for 2 h with confluently grown human pharyngeal cell line Detroit 562. Plating control samples (at 0 h and 2 h) confirmed that the applied bacteria were able to grow during the incubation period reaching titers between 10^7–10^8 CFU (data not shown). After 2 h, the Detroit cells were extensively washed, lysed and the suspensions were plated. Recovered CFU counts include bacteria that were either able to bind to the surface and invade the human cells (see Figure11A). To determine only invasive bacteria, surface bound pneumococci were killed by treatment with antibiotics prior to lysis of the eucaryotiv cells (see Figure 11B).

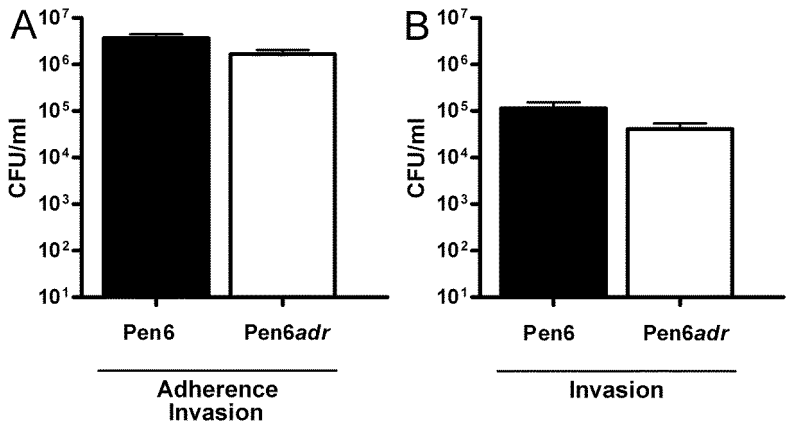

Figure 11: Adherence of Pen6 and Pen6adr to the human pharyngeal cell line Detroit 562.

10^6 CFU bacteria were incubated with Detroit 562 cells. After 2 h bacteria were recovered that **(A)** adhered and invaded the human cells, **(B)** invaded the human cells. Data from 6 independent experiments is shown.

The results suggest that a functional *adr* gene does not influence the capacity of pneumococci to attach to nasopharyngeal cells. Also similar amounts of intracellularly

protected wildtype and mutant bacteria were recovered, suggesting that also invasiveness of *S. pneumoniae* is not dependent on the presence of *O*-acetyl groups.

2.1.2.2 In vitro Growth of R36ASIII and R36ASIII*adr*

Growth capabilities of capsule type III strains R36ASIII and R36ASIII*adr* in C+Y medium were determined. The growth curves were repeated 6 times and both strains showed identical growth behaviors and doubling times (see Figure 12).

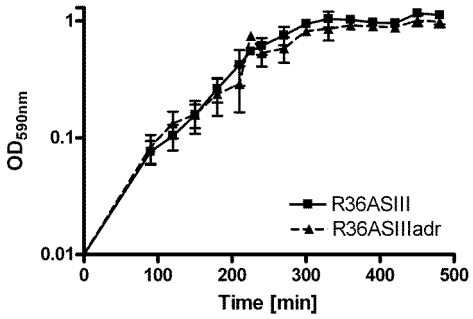

Figure 12: In vitro growth of R36ASIII and R36ASIIIadr.

Bacteria were grown in C+Y medium and optical density at 590nm was measured. Wildtype and mutant bacteria showed identical growth behavior. Data of 6 independent experiments is displayed.

2.1.2.3 Nasopharyngeal Colonization of R36ASIII and R36ASIIIadr

Mice (n=5 per strain) were anaesthetized and inoculated into the nostrils with 10^6 CFU of either strain R36ASIII or R36ASIII*adr*. After 48 h animals were sacrificed and nasopharyngeal washings were plated. A striking difference in the number of isolated, viable bacteria between the two strains was observed. Significantly (unpaired student-t test, $p<0.002$) less R36ASIII*adr* were recovered from the nasopharynx as compared to the respective wildtype R36ASIII. These data suggest that *O*-acetylation of the peptidoglycan backbone is essential for the establishment of nasopharyngeal colonization in the murine host (see Figure 13).

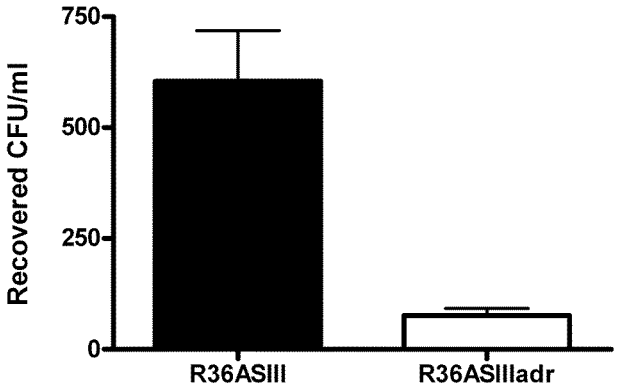

Figure 13: Nasopharyngeal colonization of R36ASIII and R36ASIIIadr.

5 mice per strain were inoculated with 10^6 CFU of either strain. After 48 h bacteria were recovered from the nasopharynx. R36ASIII*adr* significantly lost its ability to establish an infection of the nasopharynx in mice.

2.2 Choline Residues of Teichoic Acids

Chapter summary:

The second series of my studies on the effect of cell wall structure on pneumococcal virulence involved the choline-residues of teichoic acids (see Figure 14). These studies examined various aspects of choline metabolism in pneumococci. The work included (i) elucidation of the mechanism of choline-independence in strain Cho$^-$, (ii) testing the impact of these aminoalcohol residues on the pathology in pneumococcal disease in several animal models. My studies produced evidence that the choline residues at the pneumococcal surface help the bacteria to evade clearance by the immune system. Thus they indirectly contribute to a pathological, overwhelming immune response.

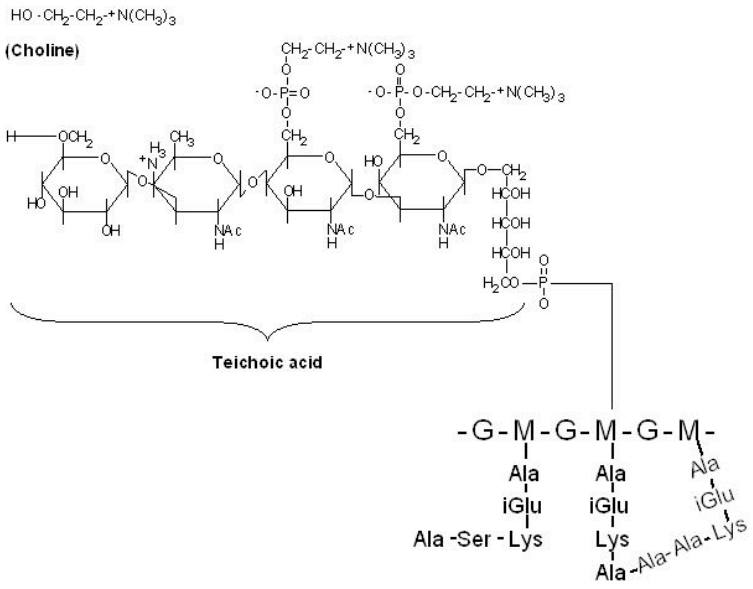

Figure 14: Chemical structure of cholinated wall teichoic acids.

Two choline residues are linked via phosphate groups to the Gal*N*Ac residues of the repeating unit of the teichoic acid backbone. The teichoic acid itself is attached via an unknown linkage unit to the Mur*N*Ac component (M) of the peptidoglycan (-G-M-G-M-).

2.2.1 Mechanism of Choline-Independence in R6Cho$^-$

*„Kharat AS, Denapaite D, **Gehre F**, Brückner R, Vollmer W, Hakenbeck R, Tomasz A. Different pathways of choline metabolism in two choline-independent strains of* Streptococcus pneumoniae *and their impact on virulence. J Bacteriol. 2008 Sep;190(17):5907-14.“*

Most of the studies on the role of choline in the biology of *S. pneumoniae* were conducted in strain R6Cho$^-$, a pneumococcal strain that was obtained by transforming R6 with *Streptococcus oralis* ATCC 35037 genomic DNA, another streptococcal species that has choline in its surface but is able to grow without choline in the growth medium [106].

Southern Blot analysis in a previous publication found that during that transformation event a SmaI recognition site in gene *spr1226* was lost in the R6Cho$^-$ chromosome, suggesting that a genetic re-arrangement of the genome could have occurred at this location [41]. To track down, locate and characterize the functions of the acquired heterologous *S. oralis* DNA elements responsible for choline-independence in the Cho$^-$ background several experiments were conducted.

2.2.1.1 Inactivation of the Wildtype lic2 operon Genes: Impact on Growth and Phenotype of R6Cho$^-$

The deletion mutants in *licD1*, *licD2* and *tacF* in the background of R6Cho$^-$ were already existent in the laboratory [27]. Cultures of the deletion mutant R6Cho$^-$Δ*licD1D2* grew in choline-containing medium in the form of diplococci or short chains, autolyzed in the stationary phase and retained sensitivity to Deoxycholate induced lysis (Table 2). To investigate the effects of a deletion of the entire *lic2* operon - including not only *licD1* and *licD2* but also *tacF* (see Figure 3 in Introduction) - strain R6Cho$^-$Δ*lic2* was analyzed. Strain R6Cho$^-$Δ*lic2* has remained fully viable and retained the normal physiology: growth in the form of autolysis-prone pneumococci with diplococcal morphology, indistinguishable from the phenotype of the parental strain

R6Cho$^-$ when grown in choline-containing medium (Table 2). These deletion experiments revealed that R6Cho$^-$ must have obtained at least two alternative heterologous elements that complement the loss of the deleted pneumococcal wildtype genes. Presumably these must encode for homologues of LicD1/2 and the teichoic acid flippase TacF.

2.2.1.2 Choline Content of the Cell walls in lic2 Mutants of R6Cho$^-$

To understand whether R6Cho$^-$ obtained one or two phosphorylcholine-transferases choline content of cell walls purified from strains R6Cho$^-$, R6Cho$^-$Δ*licD1D2* and R6Cho$^-$Δ*lic2* was determined. Each culture was grown in choline containing media, which was also supplemented with ^{3}H-choline. Results shown in Table 2 indicate that the cell walls of the R6Cho$^-$Δ*licD1D2* and R6Cho$^-$Δ*lic2* mutants had reduced choline content, corresponding to about 50% of the choline content of the parental strain R6Cho$^-$ grown under the same conditions. Apparently, this reduced choline content in the cell walls was sufficient for the cells to maintain a normal (diplococcal and autolysis prone) phenotype (Table 2).

Table 2: Impact of deletions in the lic2 operon on the physiology and phenotype of R6Cho- when grown in the presence of choline

Mutation	**none**	**Δ*licD1D2*, Δ*lic2***
Morphology	Diplococci	Diplococci
Autolysis	+	+
Choline content	100 %	50 %

2.2.1.3 Identification of the Inserted *S. oralis* DNA in Strain R6Cho$^-$

In the course of the experiments, a preliminary genome sequence of another *S. oralis* strain Uo5 [107] became available, which was very useful to provide a first understanding of the genome of donor *S. oralis* ATCC 35037. The genomic region of Uo5 corresponding to the vicinity of gene *spr1226* in R6 appeared to be homologue enough to recombine into the R6 pneumococcal genome.

In order to prove this, long range PCR experiments were performed using primers located inside a number of genes in this genomic region. Primers were designed to match to genes of both strains, R6 and *S. oralis* Uo5. Presumably these primers should also bind to the potentially integrated *S. oralis* ATCC 35037 DNA in strain R6Cho$^-$. Several PCR products were obtained by this approach all indicating that a large replacement of R6 DNA by *S. oralis* DNA occurred and led to the mutant strain R6Cho$^-$. As an example, the 13 kb product amplified from R6Cho$^-$ DNA with primers located in *hom* and *pheA* is shown in Figure 15A. The corresponding fragment of R6 is only 10 kb in size. The nucleotide sequence of this 13 kb PCR product revealed the nature of the new genes that were integrated into R6Cho$^-$ replacing endogenous R6 genes (see Figure 15B). A new copy of *licD3* was found right next to the *spr1226* homologue followed by a gene encoding a putative glycosyl transferase (*orf2*). A second *licD* gene, tentatively designated *licD4*, genes (*orf4*, *orf5*) for two hypothetical proteins, and the phosphocholine esterase gene *pce* [29] are additionally present in this locus. The LicD4 protein has a large extension of about 450 aa at its N-terminus, which is not found in other LicD proteins. And most importantly, a *tacF* homologue encoding the expected teichoic acid flippase is found downstream of *pce* and on the opposite strand (see Figure 15B).

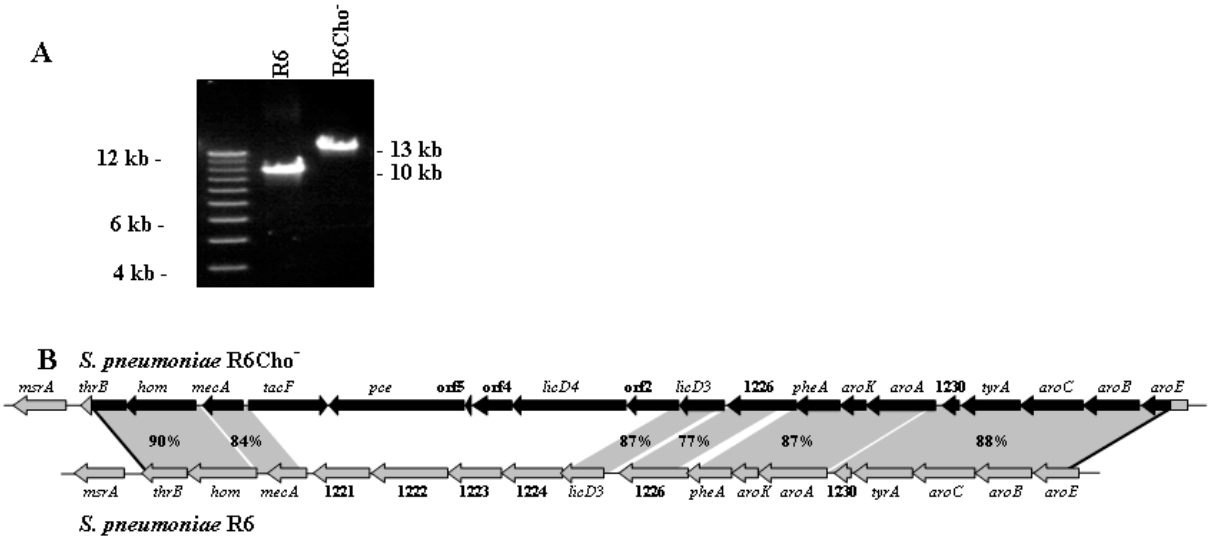

Figure 15: Schematic representation of the *licD3* locus in *S. pneumoniae* R6 and the choline-independent derivative R6Cho$^-$.

(A) PCR products amplified from R6 and R6Cho$^-$ backgrounds with primers binding to genes *hom* and *pheA*, **(B)** The genomic organization of the *licD3* region of *S. pneumoniae* R6 (bottom) is shown along with the same region of *S. pneumoniae* R6Cho$^-$ after integration of *S. oralis* ATCC35037 DNA (top). Endogenous R6 genes are shown in grey and are given by gene names or spr numbers [26,41,108]. Acquired *S. oralis* genes are shown in black. The sites of recombination are indicated by black lines. Replacement of *S. pneumoniae* R6 DNA by *S. oralis* ATCC35037 DNA occurred between *thrB* (position 1.214.973 in the R6 genome) and *aroE* (position 1.231.893). The degree of identity between *S. pneumoniae* and *S. oralis* genes is indicated. Some of the gene products, whose genes showed no significant similarity to R6, could be identified by their protein similarity. Designations *orf2, 4, 5* were given according to their position in the presumed *licD3* operon of *S. oralis*. Identities of R6 genes *spr1221-1224* to *S. oralis* genes could not be assessed since the whole genome sequence of *S. oralis* ATCC35037 is not available. The *S. oralis* nucleotide sequence is available from genbank (Accession number is EU675999).

2.2.2 Essential Role of Choline in Pneumococcal Meningitis

***"Gehre F,** Leib SL, Grandgirard D, Kummer J, Buehlmann A, Simon F, Gäumann R, Kharat AS, Täuber MG, Tomasz A. Essential role of choline for pneumococcal virulence in an experimental model of meningitis. J Intern Med. 2008 Aug; 264(2): 143-54."*

To determine the effect of choline in the pneumococcal cell wall on virulence in meningitis the infant rat model was used. Animals were infected with either D39Cho$^-$ or D39Cho$^-$licA64. Bacterial inocula were grown in choline-free Cden medium in order to assure that both strains had the same chain forming and autolysis negative phenotype at the time just prior to infection. Doses of 5 x 10^2 CFU or 2 x 10^3 CFU were injected i.c. into 11 day old WISTAR rats. Due to the *in vivo* availability of exogenous choline, D39Cho$^-$ is known to rapidly revert to a choline containing phenotype [41]. In contrast, strain D39Cho$^-$licA64, carrying the *licA* mutation, is unable to utilize exogenous choline and therefore, continues to produce choline-free cell walls and retains the chain forming and autolysis negative phenotype in the *in vivo* environment [41].

2.2.2.1 The Pathology of Meningitis is choline-dependent

2.2.2.1.1Activity score, Virulence and Bacterial Load in the CSF

The onset of infection and development of meningitis was tracked by scoring the disease severity of animals at different times after infection (Table 3) and determination of survival up to 36 h after infection (see Figure 16A).

Infection with D39Cho$^-$ induced a steady increase in the disease severity of the animals, which ended in a moribund state. Animals challenged with 2 x 10^3 CFU of strain D39Cho$^-$ died within 22 h (median survival time=18.0 h, n=16 animals).

A lower infection dose with this strain delayed death slightly (median survival time=20.0 h, n=12 animals, data not shown). In contrast, infection with the same CFU of strain D39Cho$^-$licA64 (n=16 animals) caused only slightly reduced physical activity

in the animals, which recovered completely at 24 h and eventually survived independently of the inoculum size.

Table 3: Activity score[a] of animals infected i.c. with 2 x 10^3 CFU bacteria at different timepoints.

Bacterial strain	Activity score over time[b]					
	4 h	8 h	12 h	18 h	24 h	36 h
D39Cho$^-$	5 ± 0	4 ± 0	3.9 ± 0.35	1.5 ± 0.76		
D39Cho$^-$licA64	5 ± 0	5 ± 0	4.5 ± 0.53	4.8 ± 0.46	5 ± 0	5 ± 0

[a] animals are turned on their back and scored as: 5 = normal, 4 = turns upright < 5s, 3 = turns upright <30s, 2 = does not turn upright, 1 = coma; [b] Values are means ± SD (n = 16 animals per group)

Figure 16B shows that both the D39Cho$^-$ and D39Cho$^-$licA64 strains were able to replicate after they were instilled into the CSF. Assuming the dispersal of the inocula (about 10^3 CFU) into a common CSF volume of 1 ml, the viable titer of the choline-free strain D39Cho$^-$licA64 increased from 10^3 CFU per ml to about 10^4 CFU per ml by the fourth hour after inoculation and increased further to nearly 10^5 CFU per ml by the eighth hour. After this time the titer of bacteria declined to about 10^4 CFU per ml (at 12 h) and went below detection after 18 h.

In the same experiment the titer of the choline containing strain D39Cho$^-$ increased from 10^3 CFU per ml to as high as five times 10^6 CFU per ml by the fourth hour, followed by a slower increase up to 10^7 CFU per ml between the fourth and eighth hour.

The initial burst of over 1000 fold increase in the titer of strain D39Cho$^-$ must be the composite product of two processes: rupture of the bacterial chain to single or diplococcal units due to the reincorporation of choline (a process prevented in the double mutant) and replication of the individual bacterial cells.

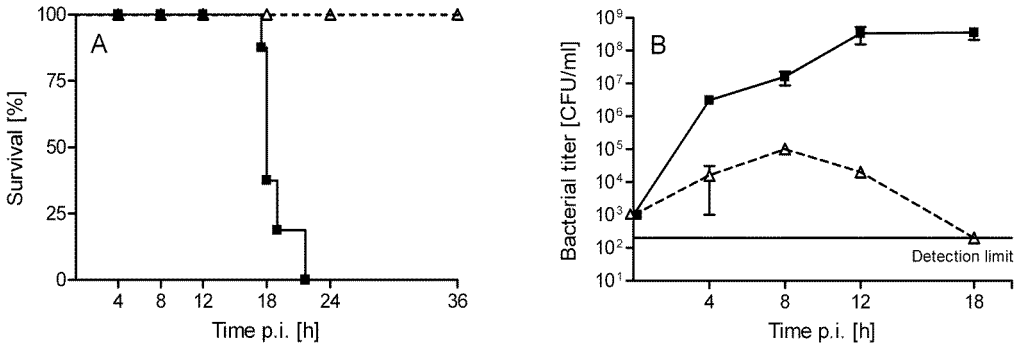

Figure 16: Survival of rats and bacterial titers in the CSF.

Rats (n=16 animals per strain) were infected i.c. with 2 x 10^3 CFU of D39Cho$^-$ (solid squares and solid lines) or D39Cho$^-$licA64 (open triangles and dashed lines). **(A)** Survival of animals was followed in the course of the infection. **(B)** Bacterial titer in the CSF at various times after infection.

2.2.2.1.2Expression of Matrix Metalloproteinase-9 (MMP-9) in the CSF

MMP-9 is an enzyme previously found to be associated with brain damage during pneumococcal meningitis [109]. Inducible MMP-9 expression was normalized against the concentrations of the constitutively expressed MMP-2. Since the levels of MMP-2 in the CSF remained unchanged during pneumococcal meningitis [109], MMP-2 was used as an internal standard. Infection with 2 x 10^3 CFU of D39Cho$^-$ (n=6 animals) or D39Cho$^-$licA64 (n=7 animals) resulted in similar production of MMP-9 in the CSF during the initial 8 h period (see Figure 17). However, rats challenged with the choline containing bacteria expressed significantly higher MMP-9 levels between 8 h – 18 h p.i. than animals infected with the choline-free mutant ($P < 0.05$, unpaired student-*t* test). A lower inoculum dose of 5 x 10^2 CFU lead to the same differential but overall weaker expression of MMP-9 (data not shown).

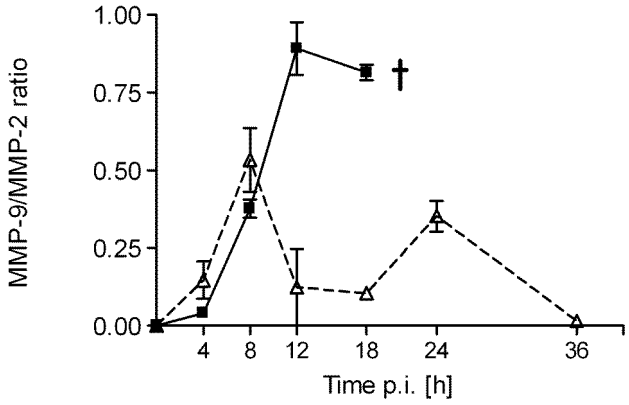

Figure 17: MMP-9 expression in the CSF.

Matrixmetalloproteinase (MMP-9) activity was measured in the CSF of animals infected with D39Cho$^-$ (n=6 animals) or D39Cho$^-$licA64 (n=7 animals).
†: indicates the death of all animals.

2.2.2.1.3Histopathology

Cortical brain damage was described to occur during bacterial meningitis [110]. Brains were collected at timepoint of death of animals infected with D39Cho$^-$ or at the end of the experiments in animals infected with D39Cho$^-$licA64. Figure 18 shows photographs of isolated and perfused brain hemispheres. A massive destruction of cortical tissue was observed after infection with D39Cho$^-$ (independent of inoculum dose), whereas D39Cho$^-$licA64 had no detectable harmful effect on the cortex by gross appearance (see Figure 18) or microscopy ($P < 0.001$, Wilcoxon signed rank test, n=25 animals per strain) (see Figure 19A,C). Pneumococcal meningitis was also shown to induce the formation of apoptotic bodies in neurons of the hippocampal dentate gyrus of infant rats [111,112]. Examination of the brains for hippocampal apoptosis showed a significant difference between rats infected with choline containing bacteria and choline-free bacteria, independent of the inoculum size ($P < 0.0001$, n=20 animals per strain) (see Figure 19 B, D). Thus, histopathology clearly showed an association between the occurrence of brain damage and the presence of choline in the cell wall of the infecting pneumococci.

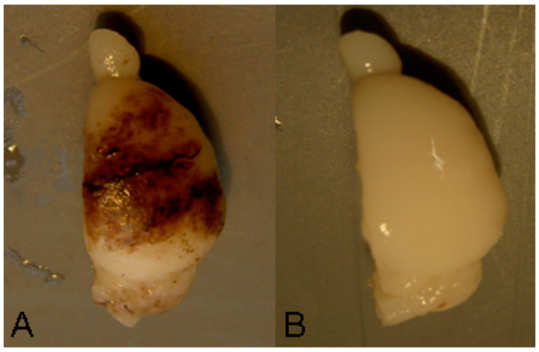

Figure 18: Brain injury due to meningitis.

The pictures show the right hemispheres of infant rat brains after infection with 10^3 CFU of **(A)** D39Cho$^-$ and **(B)** D39Cho$^-$licA64. Brains were collected at timepoint of death in case of D39Cho$^-$ and at 36 h p.i. for D39Cho$^-$licA64.

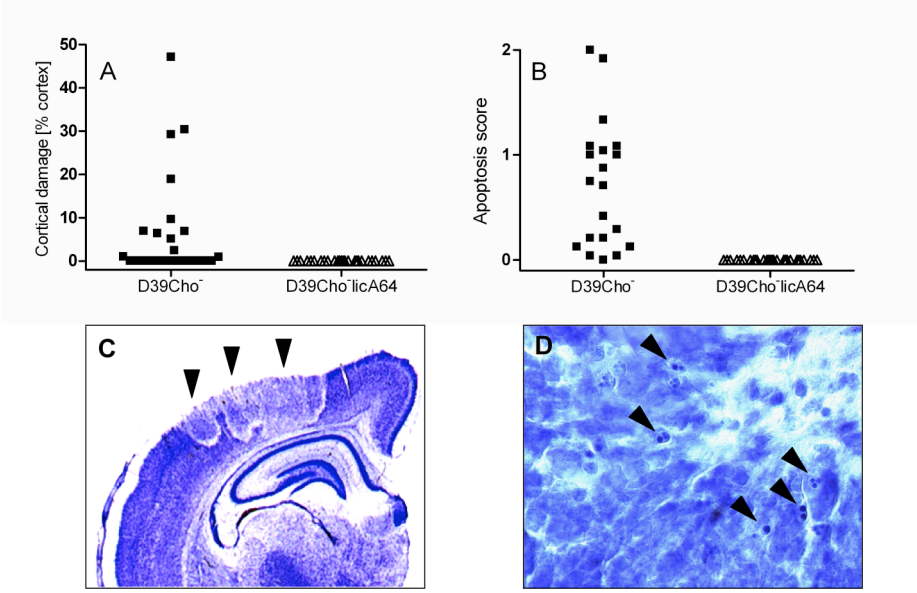

Figure 19: Cortical damage and apoptosis in the hippocampal dentate gyrus.

Rats were infected i.c. with D39Cho$^-$ (solid squares) or D39Cho$^-$licA64 (open triangles) and **(A)** the frequency of cortical damage and **(B)** the extent of apoptosis were measured. Histopathology of cortical and hippocampal injury assessed by Nissl staining (Cresyl-violet): **(C)** Cortical injury is characterized by wedge shaped areas of decreased neuronal density suggestive of ischemic necrosis. **(D)** Section of hippocampus exhibiting the presence of apoptotic nuclei (arrowheads) characterized by apoptotic bodies, the histomorphological hallmark of apoptosis in the subgranular zone of the dentate gyrus.

2.2.2.2 Inflammation during Meningitis is Choline-dependent

To test the two strains for their potential to stimulate inflammation in the CSF space 11 days old Wistar rats were infected with live bacteria. To further understand the contribution of choline residues in particular, choline containing and choline-free cell walls were injected into the cisterna magna. In both experimental setups CSF samples were collected at different timepoints and various inflammatory parameters (cytokines, neutrophil influx) were tested.

2.2.2.2.1Cytokine Production in the CSF induced by live bacteria

In order to get insight into the immune responses to D39Cho$^-$ and D39Cho$^-$licA64, cytokines were measured in CSF samples taken throughout the course of infection. Figure 20 shows cytokine concentrations in the CSF of animals that received 2 x 10^3 CFU of either D39Cho$^-$ or D39Cho$^-$licA64 bacteria (n = 16 animals per strain). Similar cytokine patterns were reproduced with inoculum sizes of 5 x 10^2 CFU (data not shown). During the first 8 h, infection with either D39Cho$^-$ and D39Cho$^-$licA64 triggered the expression of comparable levels of the pro-inflammatory cytokines IL-1β, IL-6, IL-12(p70), TNF-α and of the anti-inflammatory cytokine IL-10.

These cytokines are known to be involved in inflammation [113], blood-brain-barrier permeation and leukocyte recruitment to the site of infection [114] during meningitis.

After 8 h a further drastic increase of these cytokine levels was observed in animals infected with D39Cho$^-$ ($P < 0.05$ – $P < 0.005$), which continued up to the time of death of the animals at 20 h.

In contrast, the choline-free strain D39Cho$^-$licA64 did not stimulate further production of IL-1β, IL-6, IL-12(p70) and TNF-α beyond 8 h and the initially elevated cytokine levels eventually declined below detection. An exception to this was the concentration of IL-10, which continued to show high level of expression between 12 h and 36 h ($P < 0.05$ – $P < 0.005$) in the animals infected with the choline-free double mutant.

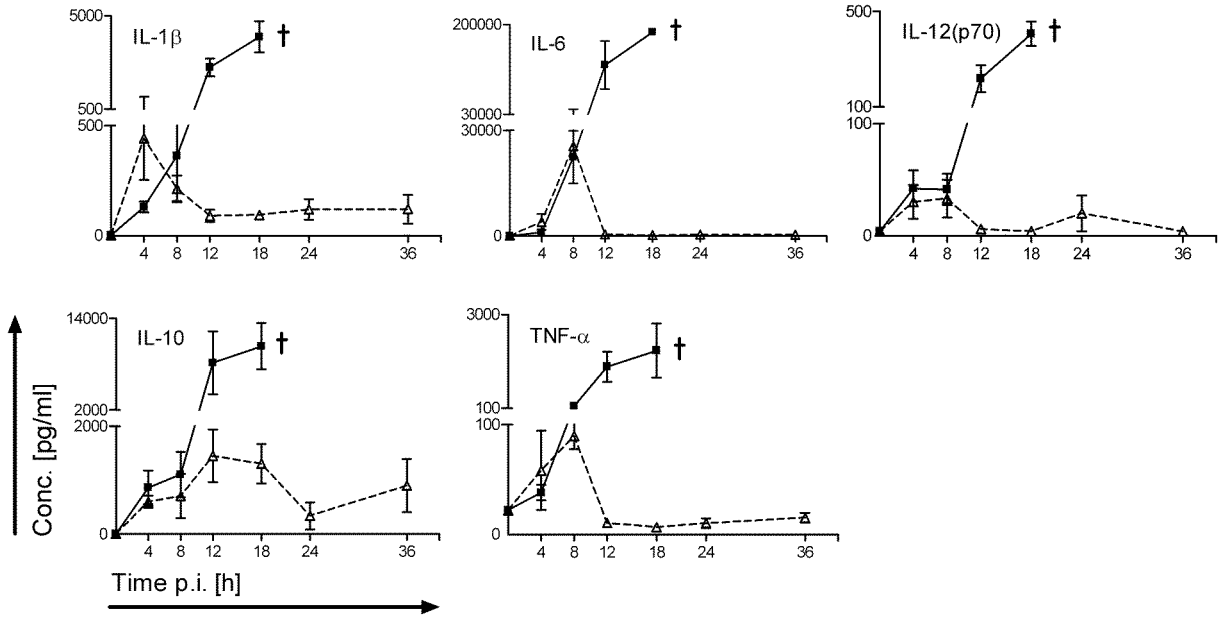

Figure 20: Cytokine expression in the CSF after infection with live bacteria.

Rats (n = 16 animals per strain) were infected i.c. with 2 x 10^3 CFU of D39Cho$^-$ (solid squares and solid lines) or D39Cho$^-$licA64 (open triangles and dashed lines) and the concentration of cytokines (pg/ml CSF) was determined at various times after infection. †: indicates the death of all animals.

2.2.2.2.2Neutrophil Influx into the CSF induced by live Bacteria

Myeloperoxidase (MPO) activity in CSF samples was measured as an index of leukocyte influx [115,116,117]. MPO is an enzyme specific for azurophil granules and represents 5 % of the total cellular protein of neutrophils [118]. Infection with D39Cho$^-$ and D39Cho$^-$licA64 resulted in increased MPO concentrations in the CSF. Figure 21A shows the time course of appearance of MPO activity in the CSF of rats after an infection with 2 x 10^3 CFU of either the choline containing or choline-free bacteria (n=16 animals per strain). Both strains showed similar increase of MPO activity in the CSF, indicating that they induced comparable neutrophil concentrations in the CSF between 8 - 12 h post-infection. Following peak concentrations at 12 h, MPO activity declined somewhat in animals infected with D39Cho$^-$ but remained high until death of the animals. In contrast, MPO activity declined sharply and became undetectable at 36 h after infection of animals with the choline-free strain D39Cho$^-$licA64.

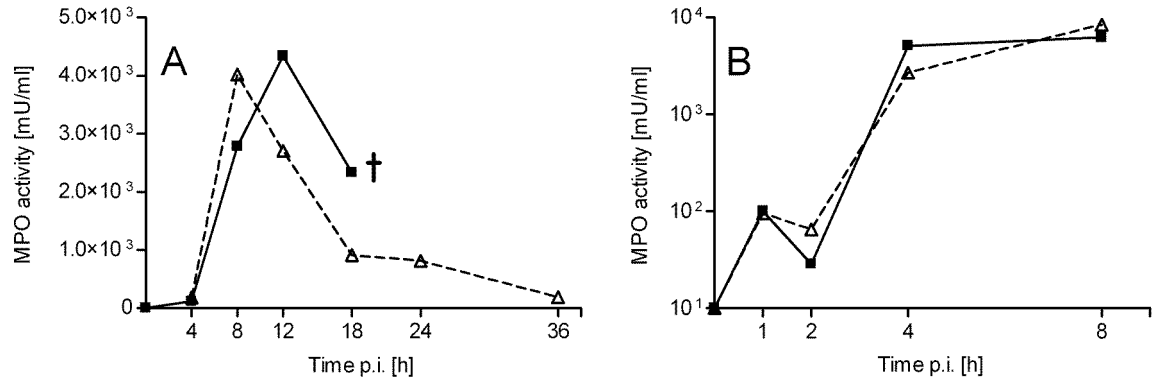

Figure 21: Neutrophil influx into the CSF.

(A) Myeloperoxidase (MPO) activity was determined in rats infected i.c. with 2 x 10^3 CFU of D39Cho$^-$ (solid squares and solid lines) or D39Cho$^-$licA64 (open triangles and dashed lines). MPO activity (in units/ml CSF) was used to estimate the influx of neutrophils into the CSF (n=16 animals per strain). **(B)** Myeloperoxidase (MPO) activity in the CSF after injection of 10^7 CFU equivalents of choline containing (solid squares and solid lines) and choline-free (open triangles and dashed lines) cell wall (n=12 animals per cell wall preparation).

2.2.2.2.3Cytokine Production in the CSF induced by cell wall preparations

Infant rats were infected i.c. with 10^7 CFU equivalents of purified cell wall preparations that differed from each other by the presence of choline residues. Cytokine production was determined at timepoints 1 h, 2 h, 4 h, and 8 h after inoculation of the cell walls (see Figure 22). Cytokine concentrations in the CSF of 3 rats per timepoint were measured. Interestingly - independent of the presence of choline - both cell wall preparation induced comparable cytokine secretion into the CSF with concentrations peaking at either 1 h-2 h (IL-10, TNF-α) or 4 h (IL-1β, IL-6), respectively, after which time cytokine levels declined to near detection levels. IL-12 (p70) was only slightly induced by either of the cell wall preparations.

2.2.2.2.4Neutrophil Influx into the CSF induced by cell wall preparations

Injection of purified cell walls with or without choline resulted in a very large increase of MPO activity in the CSF over time (see Figure 21B), which was virtually identical for choline containing and choline-free cell walls.

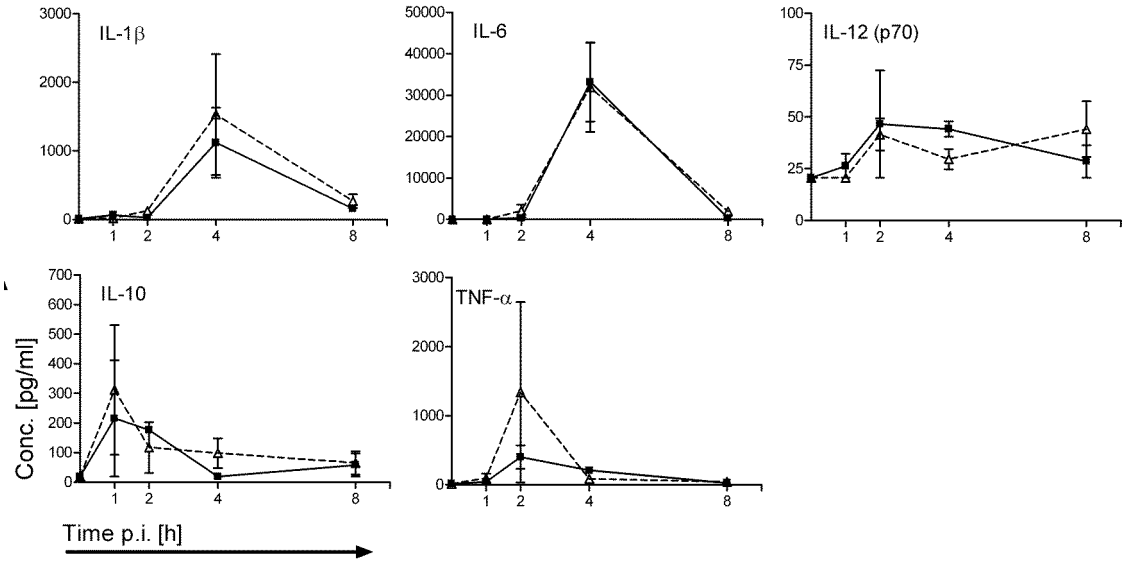

Figure 22: Cytokine expression in the CSF after injection of choline containing and choline-free cell wall preparations.

Rats were infected with 10^7 CFU equivalents of either choline containing (solid squares and solid lines) or choline-free (open triangles and dashed lines) cell wall and the concentration of cytokines (pg/ml CSF) was determined at various times (h) post infection (p.i.).

2.2.3 Essential Role of Choline in Pneumococcal Sepsis

*"**Gehre F**, Spisek R, Kharat AS, Matthews P, Kukreja A, Dhodapkar MV, Tomasz A., The role of teichoic acid choline moieties in the virulence of Streptococcus pneumoniae. Infect. Immun. 2009"*

D39Cho$^-$ and D39Cho$^-$licA64 were previously tested in a mouse sepsis model and a drastic reduction of virulence was observed during an infection with the choline-free double mutant [41].

2.2.3.1 The Pathology of Pneumococcal Sepsis is Choline-dependent

2.2.3.1.1Virulence and Bacterial Load in the Blood

Figure 23 demonstrates a repeat of this experiment: eight week old female CD1 mice were infected i.p. with 10^6 CFU of either D39Cho$^-$ or D39Cho$^-$licA64. Infection with the highly virulent D39Cho$^-$ was fatal, while D39Cho$^-$licA64 was virtually avirulent (see Figure 23A). In each of the three experiments two mice at each timepoint were sacrificed to determine bacterial load in the blood (see Figure 23B). Samples taken by heart puncture about 5 minutes after the intraperitoneal inoculation showed a comparable viable titer (about 10^4 CFU/ml blood) for both bacterial strains.

By the third hour after infection the viable titer of both D39Cho$^-$ and D39Cho$^-$licA64 had increased to about 10^7 CFU/ml blood, indicating that both the choline containing (virulent) and the choline-free (avirulent) strains were able to multiply in the blood during the first 6 hours following i.p. inoculation.

After the 6 hour timepoint the viable titer of the choline containing strain D39Cho$^-$ continued to increase steadily reaching a titer of close to 10^9 CFU/ml at the ninth hour post infection. In contrast, the titer of the choline free double mutant began to decline after the sixth hour timepoint and the bacteria were eventually cleared from the bloodstream.

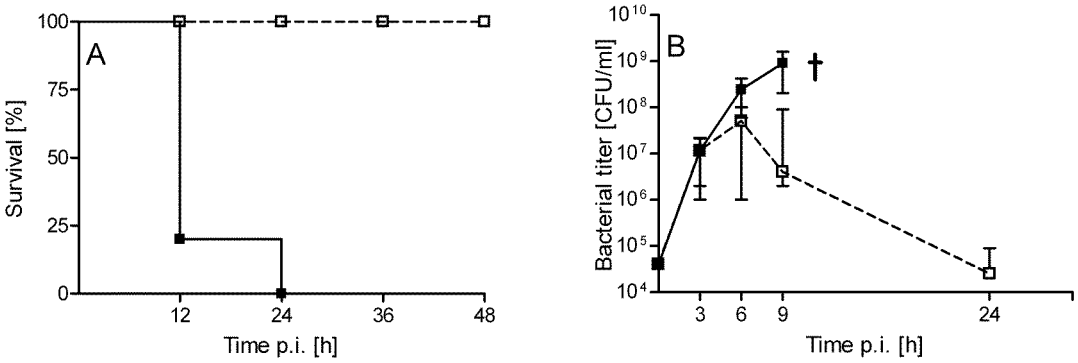

Figure 23: Survival of mice and bacterial titers in the blood.

(A): Survival of animals in the course of an i.p. infection with either 10^6 CFU D39Cho$^-$ (solid lines and solid squares) or 10^6 CFU D39Cho$^-$licA64 (dashed lines and open squares). **(B):** Bacterial titer in the blood. Mice were infected i.p. with 10^6 CFU bacteria. D39Cho$^-$ (solid lines and solid squares) were able to multiply while D39Cho$^-$ licA64 (dashed lines and open squares) were cleared from the blood. The median plus range of the bacterial growth of 3 independent experiments is displayed. †: indicates the death of the animals.

2.2.3.1.2Toll-like Receptor 2 (TLR-2) does not contribute to the Pathology

Several reports have considered the involvement of TLR-2 in the host immune response to invasion by gram positive pathogens, specifically through the recognition of structural features of the lipoteichoic acid component of the bacteria [119,120,121]. Choline is a structural component of the pneumococcal cell wall and cell membrane teichoic acids and I tested the possible role of the TLR-2 receptor in the pathophysiology of infection by the choline-containing strain.

Two types of experiments were performed. The genetic background of the TLR-2 mouse mutant is C57BL/6 which is different from the genetic background of the CD1 mouse in which all the previous virulence studies have been performed. Therefore, prior to using the TLR-2 mouse mutant I first tested the virulence properties of the choline-containing strain D39Cho$^-$ and the choline-free D39Cho$^-$licA64 in the C57BL/6 mouse background.

Infection of the C57BL/6 mice with various concentrations (n=5 animals per bacterial concentration) of either the choline-containing or the choline-free strain reproduced the same findings previously documented with the CD1 mouse: D39Cho$^-$ was highly

virulent while D39Cho$^-$licA64 was virtually avirulent in the C57BL/6 mice (data not shown).

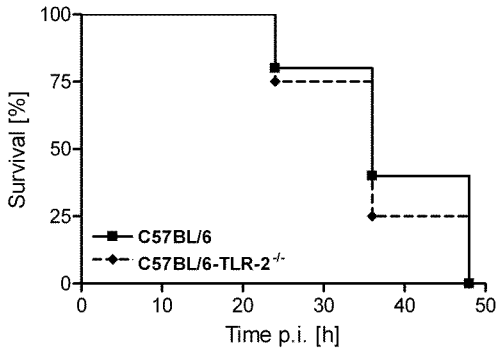

Figure 24: Effect of TLR-2 on the survival of mice.

C57BL/6 (solid lines) and C57BL/6-TLR-2$^{-/-}$ (dashed lines) (5 animals per group) were infected with 10^5 CFU of D39Cho$^-$ and survival was monitored over time.

In the second experiment I proceeded to test the possible contributions of TLR-2 to the lethality of infection by the choline containing strain D39Cho$^-$. Groups of the control mice C57BL/6 and the Toll-like receptor mutant C57BL/6-TLR-2$^{-/-}$ were infected i.p. with the choline-containing D39Cho$^-$ (5 mice per group) and mouse survival was monitored over time. There was no significant difference in the susceptibility of the two mouse strains: both showed high susceptibility to the infection, resulting in the death of all animals within 48 hours. Figure 24 shows survival curves of the tested mouse strains after an i.p. infection with 10^5 CFU D39Cho$^-$.

2.2.3.2 Inflammation during Murine Sepsis is Choline-dependent

2.2.3.2.1Cytokine Production in the Serum

There was a potent and quantitatively comparable production of the pro-inflammatory cytokines IL-1β, IL-6, IL-12(p70), TNF-α and IFN-γ stimulated by both strains during the initial 6 h time period following inoculation (see Figure 25). Although the high range in the expression of some cytokines (e.g. IL-6) suggests that some mice might

be more affected by the infection with D39Cho$^-$licA64 than others, none of the animals showed obvious phenotypical symptoms of pneumococcal disease.

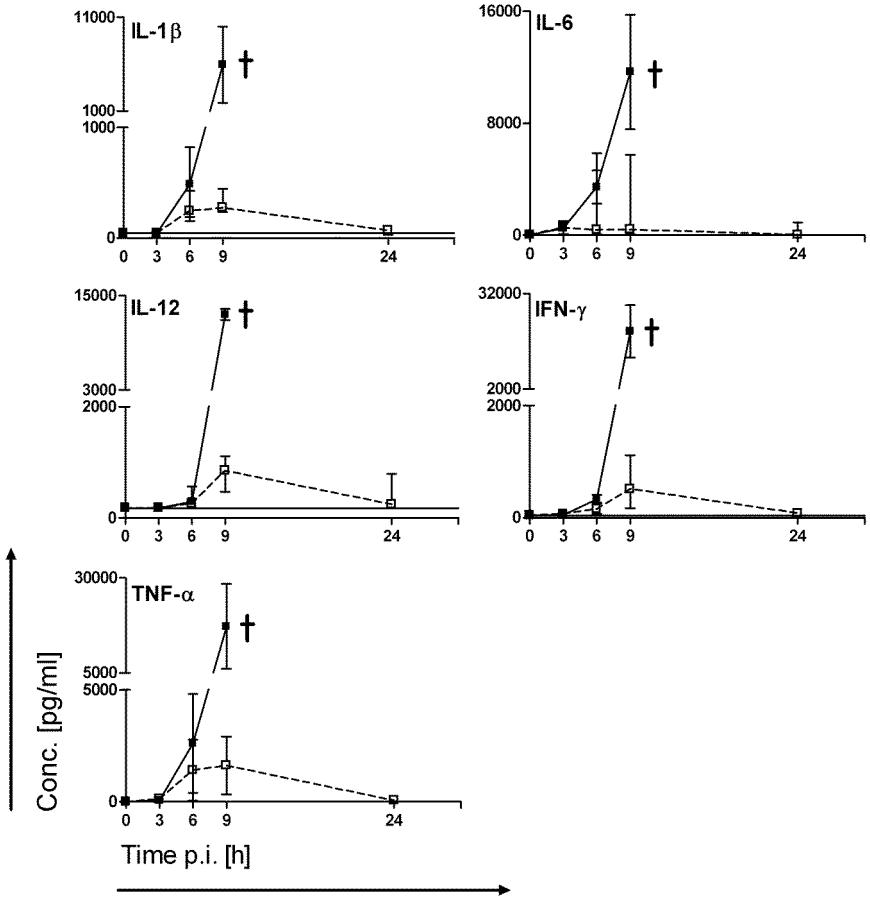

Figure 25: Time course of cytokine expression in the serum.

In each experiment mice (2 animals for each time point) were infected i.p. with 10^6 CFU of D39Cho$^-$ (solid lines and solid squares) or D39Cho$^-$licA64 (dashed lines and open squares). The production of IL-1β, IL-6, IL-12(p70), TNF-α and IFN-γ was monitored over time. The median plus range from three independent experiments is shown. †: indicates the death of the animals. The solid line indicates cytokine expression in naïve serum.

However, after this timepoint, cytokine levels induced by the two strains began to differ: there was a continued production of pro-inflammatory cytokines in parallel with the increase in the titer of the choline-containing D39Cho$^-$ cells in the blood. In contrast and consistent with their clearance from the blood, the choline-free double mutant D39Cho$^-$licA64 did not induce further expression of cytokines between 6 and 24 hours after infection. Figure 25 shows the time course of cytokine production from three independent experiments. The cytokines GM-CSF, IL-2, IL-4, IL-5 and IL-10

were also tested in the assay but their titers were below detection limits and are not shown in the figure.

2.2.3.2.2Maturation of Splenic Dendritic Cells

Spleen derived dendritic cells from mice infected with either D39Cho$^-$ or D39Cho$^-$licA64 were compared to dendritic cells isolated from healthy CD1 control mice. Both bacterial strains caused comparable expression patterns of costimulatory molecules on dendritic cells (Figure 26). CD11c$^+$CD8$^+$ dendritic cells showed upregulation of both of the tested costimulatory molecules at 6 h and 9 h after the infection. On CD11c$^+$CD8$^-$ dendritic cells only CD86 was induced at these timepoints. The results indicate that both bacterial strains were able to stimulate dendritic cell maturation to a comparable degree.

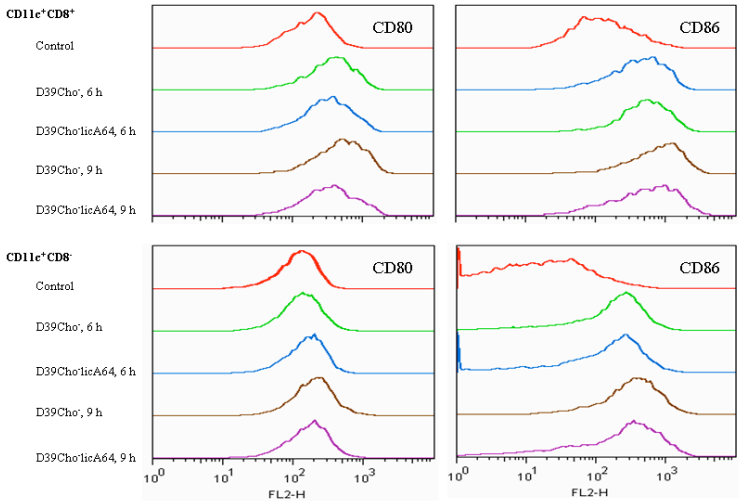

Figure 26: Maturation of murine splenic dendritic cells in vivo.

CD11c$^+$CD8$^+$ and CD11c$^+$CD8$^-$ dendritic cells, isolated from the spleen were analyzed for the presence of CD80 and CD86. Dendritic cells from a control mouse and from mice infected i.p. with 10^6 CFU of either D39Cho$^-$ or D39Cho$^-$licA64 were compared at 6 h and 9 h after infection.

2.2.3.2.3In vitro Maturation of human Monocyte-derived Dendritic Cells

When human monocyte derived dendritic cells (10^6) were incubated with 10^8 cell equivalents of Mitomycin C-killed cells of either the choline-containing or the choline-

free strain, upregulation of the co-stimulatory molecules was detected, as compared to controls of immature and/or fully matured LPS treated dendritic cells. Evidence for the maturation of human dendritic cells was shown by the expression of CD80, CD83 and CD86. However the choline-containing bacterium was a slightly stronger inducer (see Figure 27). Most interestingly, cell walls prepared from either the choline-containing or the choline-free bacteria used at concentrations representing 10^8 cell equivalents, were both able to induce comparable expression of the same dendritic cell markers (see Figure 27).

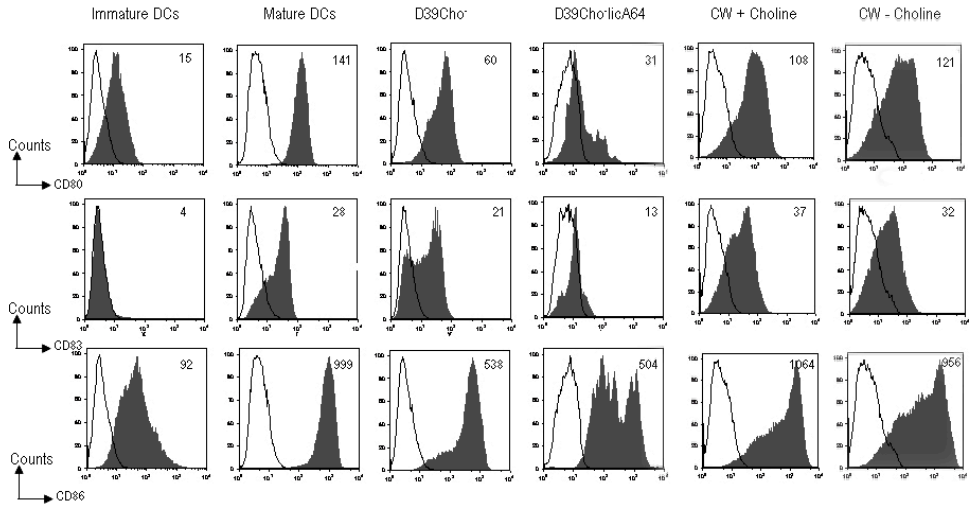

Figure 27: Maturation of human monocyte-derived dendritic cells *in vitro*.

Immature DCs were cultured for 36 h with either 10^8 CFU equivalents of Mitomycin C-killed D39Cho$^-$ and D39Cho$^-$ licA64 or 10^7 CFU equivalents of purified cell walls with or without choline (CW+/- choline) or left untreated. LPS matured (LPS-treated) DCs were used as positive control. Cells were harvested and stained for cell surface expression of CD80, CD83 and CD86. Data shown are gated for live population. The numbers represent the mean fluorescence intensities. Open histograms represent isotype controls.

2.2.4 The Role of the Choline residue in Bacterial Growth within the Host

> ***"Gehre F**, Tomasz A. Choline-dependent resistance of Streptococcus pneumoniae against murine serum and the cationic antimicrobial peptide Nisin can be abolished by C-reactive protein and TEPC-15 IgA antibodies. Manuscript in preparation, 2009"*

2.2.4.1 In vivo growth of choline-free S. pneumoniae in the Murine Host

For the time course studies of bacterial blood titers, CD1 mice were infected i.p. with 1 x 10^4 CFU of D39Cho$^-$licA64. Seven mice at each time point (6 h and 9 h) were sacrificed to determine bacterial load in the blood (see Figure 28A). The choline-free D39Cho$^-$licA64 was able to establish a transient infection and multiplied in the bloodstream during the first 6 h following inoculation. At 6 h maximal blood titers of 1.4 x 10^6 CFU/ml were reached, followed by a 10-fold decline of viable counts down to ~10^5 CFU/ml within the next three hours.

2.2.4.2 Surface-bound Choline protects S. pneumoniae against the antimicrobial Activity of Murine Serum

The next step was to understand whether an antimicrobial component of the serum itself is responsible for the observed clearance of D39Cho$^-$licA64. I sought to test the antimicrobial activity of serum taken at time point 9 h p.i. from mice infected with choline-free bacteria. To understand the serum-induced killing three different approaches were used to manipulate choline content of *S. pneumoniae*: One was to use an isogenic, genetically mutated strain D39Cho$^-$licA64 that is able to produce a bacterial cell surface free of choline thus showing the phenotypes of chain formation and deficiency of the autolytic system. In a second and third setup, surface-exposed choline residues of strain D39Cho$^-$ were either blocked by a choline-specific IgA antibody TEPC-15 or by C-reactive protein (CRP), respectively.

Reinfection of the serum *ex vivo* with 1 x 10^4 CFU/ml of the choline-free D39Cho$^-$licA64 showed that the bacteria grew for the first three hours, reaching 10^5 CFU/ml. After that, the antimicrobial activity of the serum started to effectively contain and eradicate the bacteria (see Figure 28B).

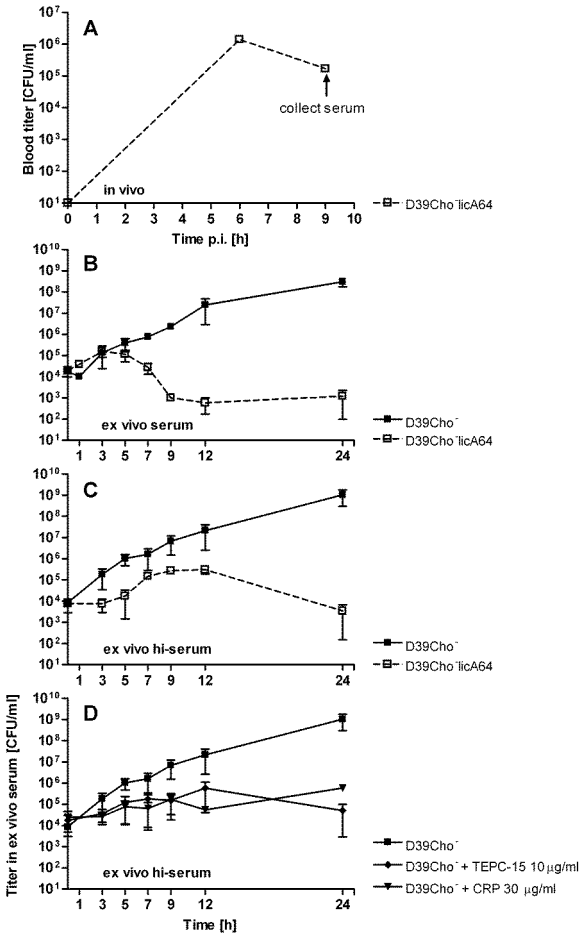

Figure 28: Choline residues protect S. pneumoniae against the antimicrobial activity of murine (complement-free) serum.

(A) *In vivo* growth of choline-free D39Cho$^-$licA64 in mice. Serum was collected at 9 h p.i. **(B)** Growth of D39Cho$^-$ and D39Cho$^-$licA64 in serum *ex vivo*. **(C)** Growth of D39Cho$^-$ and D39Cho$^-$licA64 in heat-inactivated, complement-free serum (hi-serum) *ex vivo*. **(D)** Effect of IgA TEPC-15 or human C-reactive protein (CRP), respectively, on the growth of D39Cho$^-$ in complement-free hi-serum *ex vivo*. All experiments were repeated three times.

In sharp contrast, when inoculated into *ex vivo* serum, the choline-containing, isogenic strain D39Cho$^-$ was resistant and multiplied to titers as high as 10^8 CFU/ml (see Figure 28B). Interestingly, we found the same, differential growth patterns of the

two bacteria in complement-free serum (see Figure 28C). Blocking of the choline residues with either IgA TEPC-15 or CRP resulted in a 1000-10000 fold decrease in growth of D39Cho$^-$ by complement-free serum (see Figure 28D).

2.2.4.3 Blocking of Choline Residues and Impact on Pneumococcal Physiology and Deoxycholate-induced lysis

D39Cho$^-$ was grown in C+Y in the presence of either 10 µg/ml TEPC-15 or 30 µg/ml human CRP. Blocking of choline residues did not impact on the growth behavior since all cultures showed identical doubling times, independent of the presence of choline-specific immune molecules (see Figure 29A). Light microscopy revealed that all bacterial preparation grew in the typical, diplococcal phenotype (data not shown). Also sensitivity towards DOC induced lysis was not altered by the blocking of choline-residues (see Figure 29B) as compared to the resistant strain D39Cho$^-$licA64.

2.2.4.4 Impact of 50% Choline-content on the Colonizing Capacity of D39Cho$^-$ Mutants

Strains D39Cho$^-$, D39Cho$^-$Δ*licD1D2* and D39Cho$^-$Δ*lic2* were grown in choline containing media to OD_{590nm}=0.6. D39Cho$^-$Δ*licD1D2* and D39Cho$^-$Δ*lic2* were shown to express only 50% of choline residues on their surface (see Table 2). Suspensions of bacteria (10 µl) at a concentration of 10^8 CFU/ml were inoculated into the nostrils and mice were sacrificed after 48h.

The 50% reduction in the amount of surface bound choline in strains D39Cho$^-$ Δ*licD1D2* and D39Cho$^-$Δ*lic2* had little effect on the bacterial physiology as such (see Table 2). However, the colonizing capacity of these mutants was reduced significantly (one way ANOVA test, $p < 0.002$) to 25% and 28% of the controls, respectively (see Figure 30).

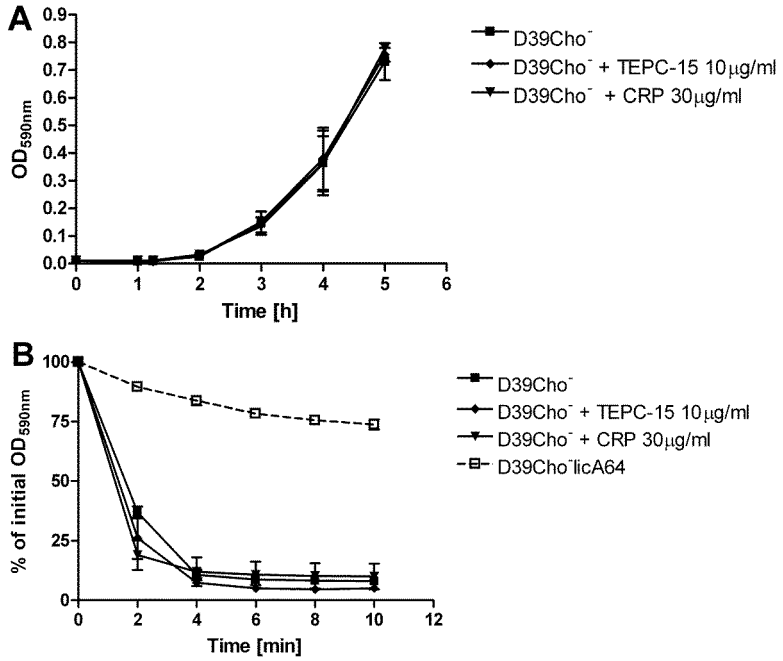

Figure 29: Blocking of choline residues and impact on the physiology of S. pneumoniae.

D39Cho$^-$ was grown in C+Y in the presence of 10µg/ml TEPC-15 or 30µg/ml human CRP. **(A)** Diplococcal shape and growth of bacteria was not affected as measured by increase of optical densities. **(B)** Independent of blocked choline residues, D39Cho$^-$ showed Deoxycholate (DOC)-induced lysis. D39Cho$^-$licA64 was resistant to DOC treatment. All experiments were repeated four times.

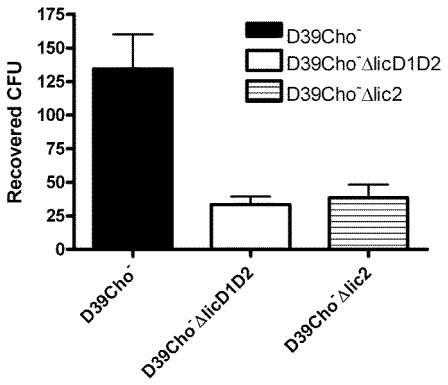

Figure 30: Effects of mutations in the lic2 operon and 50% surface-bound choline on the colonizing capacity of D39Cho-.

Strains D39Cho$^-$, D39Cho$^-$Δ*licD1D2* and D39Cho$^-$Δ*lic2* were tested and compared in the mouse nasopharyngeal colonization assay. Bacteria recovered from nasal washings were plated and enumerated.

2.2.4.5 Nisin-resistance of S. pneumoniae is Dependent on Surface-bound Choline

The susceptibility of strain D39Cho$^-$ against the cationic antimicrobial peptide Nisin was tested. My data showed that almost 60% of D39Cho$^-$ were protected against killing in regards to respective controls (see Figure 31A). Although Nisin induces lysis of TEPC-15/CRP treated D39Cho$^-$ (see Figure 31A), the results demonstrate that the antimicrobial activity of this peptide does not necessarily require a functional autolytic system as seen by the killing of D39Cho$^-$licA64 (see Figure 31B). However, in all three experiments, in which choline content was reduced, bacteria experienced 10fold enhanced Nisin-induced killing and impairment of protection leading to a decrease in viable counts (see Figure 31B). In an additional experiment using the isogenic pair of choline-containing D39Chi and choline-free D39ChilicA65 [28] the loss of surface-bound choline lead to an even more drastic, 100 fold reduction of protection against Nisin (data not shown).

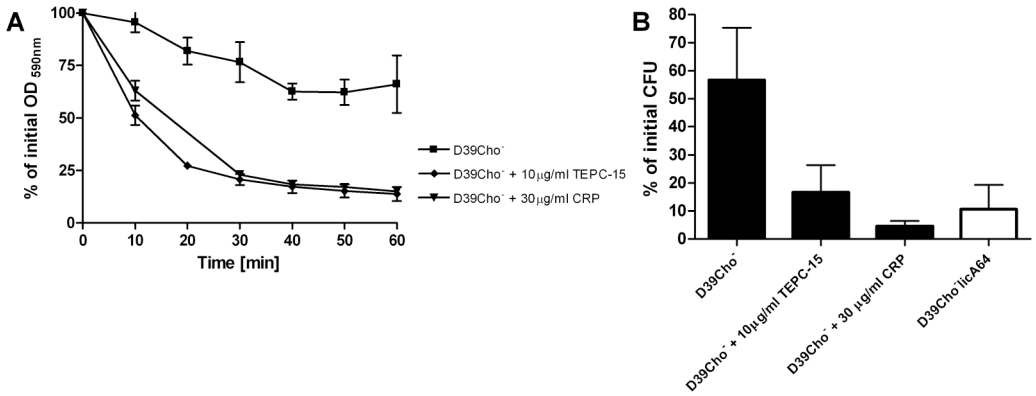

Figure 31: Surface-bound choline residues protect *S. pneumoniae* against the antimicrobial activity of Nisin.

Bacteria D39Cho$^-$ and D39Cho$^-$licA64 were grown until OD_{590nm}=0.3 and treated with 1 µg/ml Nisin. Prior to lysis cultures remained untreated or were incubated with IgA TEPC-15 or human C-reactive protein (CRP): **(A)** Nisin-induced lysis was determined by measuring OD_{590nm}. **(B)** Viable counts of bacteria at 60 min after Nisin addition. All experiments were repeated three times.

2.2.4.6 Choline-specific Immune Molecules protect Mice against Infection with S. pneumoniae

To see whether IgA TEPC-15 or CRP can protect animals, mice were immunized i.p. with 200 µg of either IgA TEPC-15 or CRP 1 h prior to infection with 3 x 10^3 CFU of the highly virulent D39Cho$^-$. At this infection dose naïve mice were shown to be dead within 36 h [41]. This was confirmed by infecting two untreated control mice. Both immune molecules reduced virulence (Logrank Test, $p < 0.03$) of the choline-containing strain and prolonged animal survival time (see Figure 32).

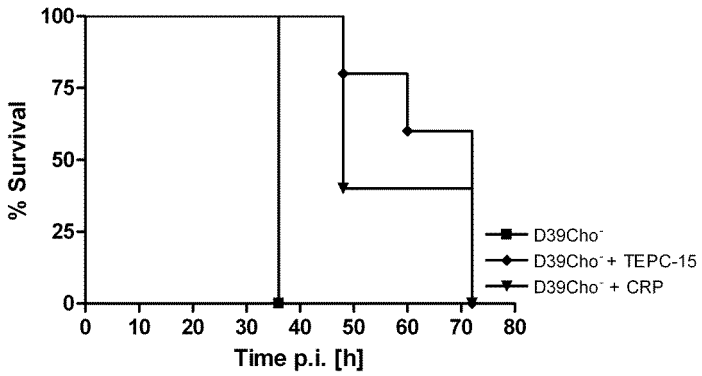

Figure 32: Choline specific immune molecules protect mice against bactereamia with *S. pneumoniae*.

Mice (n=5) were treated with 200 µg of IgA TEPC-15 or human C-reactive protein (CRP), respectively, 30 min prior to an intraperitoneal infection with 10^3 CFU D39Cho$^-$. Blocking of choline residues *in vivo* lead to prolonged survival of passively vaccinated mice. Two control mice confirmed the proper death of untreated animals within 36 h.

To test the observed effect on mucosal colonization 10^7 CFU of the choline containing strain D39Cho$^-$ were coadministered with 2,5 µg of either TEPC-15 or CRP into the nasopharynx of mice. In parallel control mice were infected with untreated D39Cho$^-$, out of which one died in the course of the infection. Bacteria in which surface-bound choline was blocked significantly (one way ANOVA test, $p < 0.05$) lost their ability to colonize the nasopharynx by 95% in respect to untreated control bacteria (see Figure 33).

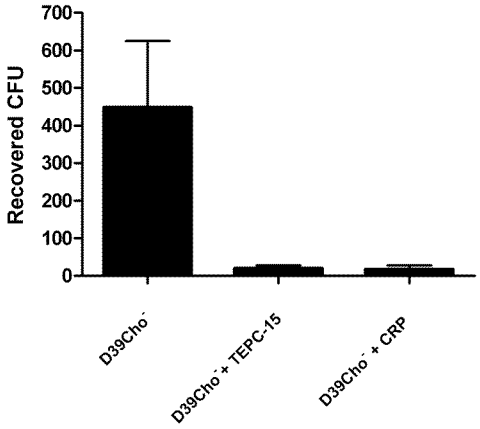

Figure 33: Choline specific immune molecules protect mice against nasopharyngeal colonization with S. pneumoniae.

Mice (n=5) were treated with IgA TEPC-15 or human C-reactive protein (CRP), respectively, prior to nasopharyngeal colonization with 10^7 CFU D39Cho$^-$. Viable bacteria were recovered 48 h after infection by nasopharyngeal washings. Choline specific immune molecules significantly reduced colonization of the nasopharynx.

2.2.5 The Protective Potential of Choline-free Strains

*"**Gehre F**, Spisek R, Kharat AS, Matthews P, Kukreja A, Dhodapkar MV, Tomasz A., The role of teichoic acid choline moieties in the virulence of* Streptococcus pneumoniae*. Infect. Immun. 2009"*

2.2.5.1 Induction of Protective Immunity by avirulent Choline-free Pneumococci

Two groups of mice (5 animals per group) were inoculated i.p. either with 10^3 CFU of the choline-free (avirulent) strain D39Cho$^-$licA64 or with 0.5 ml saline in the control group. On day 10 post inoculation all the surviving mice were challenged i.p. with 10^4 CFU of the choline-containing (highly virulent) strain D39Cho$^-$. All 5 animals from the saline control group died within 48 hours after challenge, whereas 4 of the 5 animals inoculated with the choline-free (avirulent strains) survived for at least 25 days of the follow up period without showing any signs of disease.

In a follow-up experiment another choline-free and avirulent *S. pneumoniae* strain D39ChilicB31 [27] was used instead of D39Cho$^-$licA64. A group of 16 mice, each inoculated with 10^4 CFU of D39ChilicB31, was distributed into four groups (4 animals per group) which were challenged on day 10 after inoculation by either one of two highly virulent *S. pneumoniae* strains: strain D39 (capsular type II) or strain SV36 (capsular type III). The two challenge strains were introduced at either 10^4 or 10^6 CFU per mouse, using four animals for each inoculum concentrations. All mice challenged with the capsular type III strain SV36 died within 48 hours after inoculation. In contrast, only one of the four mice challenged with the capsular type II strain D39 at 10^4 CFU per animal died and all four animals challenged with strain D39 at 10^6 CFU survived.

On day 25 post immunization the seven mice surviving the D39 challenge were next inoculated with 10^4 CFU of the capsular type III strain SV36. All seven mice died within 2 days after the challenge (see Table 4).

Table 4: Vaccination with the choline-free strain D39ChilicB31 induces serotype-specific protection.

Day 1	Day 10		Day 25	
Immunize with 10^4 CFU D39ChilicB31	Challenge with	Survival	Challenge with	Survival
4 mice →	10^4 CFU D39 →	3/4 →	10^4 CFU SV36 →	0/3
4 mice →	10^6 CFU D39 →	4/4 →	10^4 CFU SV36 →	0/4
4 mice →	10^4 CFU SV36 →	0/4		
4 mice →	10^6 CFU SV36 →	0/4		

2.2.5.2 Production of capsule specific antibodies

Eight mice were infected with 10^4 CFU of D39ChiplicB31. This strain expresses the capsular polysaccharide II on its surface. On day 10 post vaccination sera were collected and the presence of capsule specific antibodies was determined by ELISA. Compared to the control sera, I found a significant increase ($p < 0.05$, students t test) in IgM antibody production specific to purified capsular polysaccharide II in mice that were previously immunized with strain D39ChiplicB31. In the same sera I did not detect any antibodies specific to capsular polysaccharide III (see Figure 34 A,B).

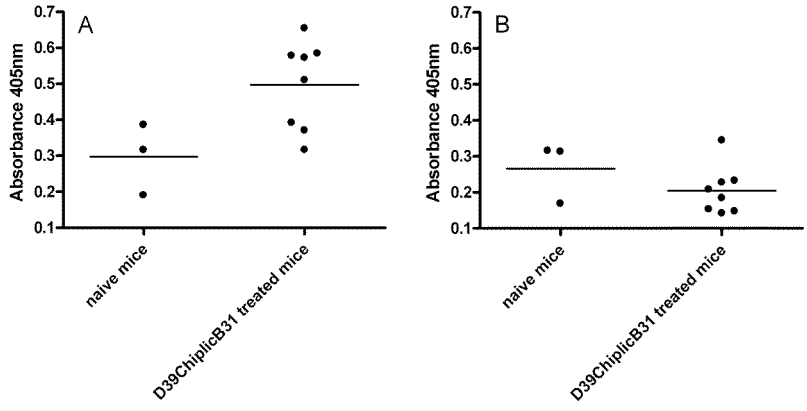

Figure 34: Detection of capsule-specific IgM antibodies with ELISA.

Mice were vaccinated with capsule II strain D39ChiplicB31. Individual sera were collected at day 10 p.i.. Sera of 8 vaccinated mice were compared to 3 naïve control sera for the production of IgM antibodies specific to (A) pneumococcal capsular polysaccharide II, and (B) pneumococcal capsular polysaccharide III.

3 Discussion

S. pneumoniae can infect various host tissues such as the respiratory tract, blood-stream and the brain. In all these infection sites the pathogen exposes its surface to the host immune system. In case of gram-positive bacteria the outermost structure is the cell wall. The aim of this thesis was to investigate on how two modifications of the pneumococcal cell wall influence the virulence of the bacterium.

Preliminary experiments showed that the deletion of the gene *adr* resulted in a loss of lysozyme resistance. The first part of the thesis was dedicated to analyse the function of this gene. I demonstrated that *adr* encodes for an *O*-acetyl-transferase, which acetylates the Mur*N*Ac residues of the peptidoglycan. Subsequent *in vitro* and *in vivo* studies showed the importance of this enzyme for the ability of *S. pneumoniae* to colonize the murine nasopharynx.

In the second part of the thesis I found that cholination of the cell wall, in particular of teichoic acids, is essential for the virulence of the pneumococcus in several known modes of infection. Choline-free bacteria were neither able to colonize the nasopharynx nor to establish bacteraemia and meningitis. Upon invasion cholinated bacteria induced a pathological proinflammatory immune response in various animal models. Despite their rapid clearance by the host, choline-free bacteria were still able to engage the host immune system to a certain degree. This limited immune response was potent enough to induce a protective immunological memory against isogenic, virulent strains of *S. pneumoniae*.

In the last part of the thesis I focused on the role the choline residue plays in evading the host immune response. The aforementioned experiments suggested that these residues might confer resistance against a host clearance mechanism. I found that cell-wall bound choline residues protect the bacterium against an intrinsic serum factor, which could be cationic antimicrobial peptides. Blocking of the choline moieties with host immune molecules abolished this immune evasion strategy of the pneumococcus.

3.1 O-Acetylation of Peptidoglycan

The use of mariner transposon mutagenesis recently identified gene *adr* [104]. It was shown that this protein confers penicillin and lysozyme resistance to strain Pen6 [104]. Sequence data of this gene revealed similarity to an *O*-acetyl-transferase of *Staphylococcus aureus* [122]. In the first part of my thesis I aimed to further characterize the effect of Adr on the molecular structure of the peptidoglycan. After that it was interesting whether Adr has an impact on the colonizing capacities of *S. pneumoniae*.

3.1.1 Adr catalyzes the *O*-Acetylation of the Peptidoglycan

To understand whether Adr is a functional *O*-acetyl-transferase the cell wall composition of strain Pen6 and the respective mutant Pen6*adr* was biochemically analyzed. Since *O*-acetyl groups are alkaline labile they were never detected in cell wall purifications that followed the conventional protocol. This conventional procedure includes buffers that are above pH7. To preserve the *O*-linked acetyl groups, the protocol used in this thesis was adjusted accordingly.

Muropeptides were prepared from highly purified cell wall isolations and analyzed by HPLC. In contrast to Pen6*adr*, Pen6 possesses two additional muropeptide peaks in the HPLC elution profiles at around 70 min. These peaks were presumably *O*-acetylated muropeptides. Compared to non-acetylated muropeptides, acetylation of muropeptides would increase their hydrophobicity and delay their retention times from a hydrophobic column. Since alkaline conditions remove *O*-acetyl groups, muropeptides of Pen6 lost these two peaks upon NaOH treatment (see Figure 8). Consequently, acetate could also be released by NaOH from whole cell wall preparations of Pen6 but not from Pen6*adr* cell wall (see Figure 10). Collecting and analyzing the various HPLC fractions by mass spectroscopy confirmed the predicted *O*-acetylated muropeptides. I found that especially Mur*N*Ac residues are the target for Adr and this modification (see Figure 9).

I also found that Adr does not influence crosslinking, since no difference in the composition of stempeptides between Pen6 and Pen6*adr* was observed (data not shown).

It was already demonstrated that *adr* mutants have increased susceptibility to lysozyme [104]. Lysozyme is a glycan hydrolase that targets the glycan strands of the peptidoglycan molecule. The enzyme binds to hexasaccharide subunits of the peptidoglycan. Upon successful binding, the β(1→ 4) glycosidic bond between the Mur*N*Ac residue in position 4 (in the hexasaccharide) and the Glc*N*Ac residue in position 5 (in the hexasaccharide) is cleaved. This specific chemical reaction demands a defined orientation and binding of the substrate. The stringent alignment of the hexasaccharide subunit fosters straining of the substrate, particularly of the Mur*N*Ac residues in position 4. This straining is the prerequisite for the successful hydrolysis of the glycosidic bond and lysis of peptidoglycan [123]. The perfect orientation of the peptidoglycan in the active crevice of lysozyme is determined by binding pockets of the enzyme that recognize de-acetylated Mur*N*Ac residues [123]. Therefore attachment of *O*-acetyl-groups to the Mur*N*Ac residues sterically interferes with lysozyme binding and results in lysozyme-resistance of wildtype pneumococci Pen6. In contrast, Pen6*adr* lost this acetylation defense strategy and can be bound and hydrolyzed by lysozyme.

Interestingly, the presence of acetyl-groups on Glc*N*Ac residues is another motif recognized by lysozyme and also required for the enzyme-substrate complex. *S. pneumoniae* was shown to secondarily remove these residues from its Glc*N*Ac residues and peptidoglycan with an N-acetylglucosamine-deacetylase PgdA [12] and therefore possesses an alternative lysozyme resistance mechanism.

3.1.2 The Impact of *O*-Acetylation on Nasopharyngeal Colonization

The first step of pneumococcal disease is the colonization of the nasopharynx. One of the major innate immune molecules in this host compartment is lysozyme. Therefore it was interesting whether the deletion of *adr* will impact the colonizing capacities of the bacterium. Since Pen6 is a non-encapsulated, non virulent strain, the *adr* mutation was tested in strain R36ASIII, expressing a polysaccharide III capsule. The lack of *O*-acetyl-groups led to decreased colonization by strain R36ASIII*adr* when inoculated in the mouse model (see Figure 13). A variety of parameters could influence the colonizing capacity.

For example, a slower growth of mutant bacteria could be a disadvantage and lead to enhanced clearance by the immune system. This does not seem to occur, since both strains R36ASIII and R36ASIII*adr* showed identical growth rates when cultivated in C+Y (see Figure 12).

Also, lack of *O*-acetylation may somehow influence the adhesion to nasopharyngeal cells in multiple ways. The acetyl groups could either function as adhesive structures themselves or their absence could indirectly influence already known adhesion molecules. To exclude that we compared the capacity of both strains to attach to, and invade human pharyngeal Detroit 562 cells *in vitro*. These experiments demonstrate that Adr does not play a role with the actual adherence / invasion process (see Figure 11). The limited capacity of *adr* mutants to colonize the murine nasopharynx appears to be rather due to an immune clearance mechanism than an adherence impairment.

Consistent with the previous findings that Adr confers lysozyme resistance to the bacterium, the observed reduction of recovered bacteria was therefore expected. Confirming that, a *pgdA* mutant in the same capsular III background, that also lacks a lysozyme resistance mechanism showed a comparable loss of colonization capacity (data not shown).

However, studies using a TIGR4 (capsule type 4) isolate demonstrated that *adr* deletion only has an impact on colonization in conjunction with a second knock-out of the *pgdA* gene [124]. One reason could be that Adr in TIGR4 is not as active as in strain R36ASIII. The authors of this study did not biochemically assess to what extent strain TIGR4 actually *O*-acetylates its peptidoglycan [124].

Additionally, I think there could also be another explanation to this obvious difference: type IV capsular polysaccharides are covalently attached to the cell surface, whereas the type three capsule of R36ASIII is non-covalently linked. It is possible that only this non-covalent attachment of type III polysaccharides is somehow dependent on the O-acetyl-groups of the underlying peptidoglycan. A potentially altered type III capsule could influence the formation of a lysozyme-peptidoglycan complex and lysozyme resistance of *adr* mutants.

One could therefore conclude, that besides the direct effect that O-acetylation has on peptidoglycan recognition by lysozyme, a secondary effect on lysozyme-resistance determined by the degree of capsule type III attachment is also conceivable.

3.2 Choline Residues of Teichoic acids

The work on the cholination of *S. pneumoniae* focused on several aspects. First it was very interesting to define the underlying genetics of the Cho$^-$ background and the mechanism of choline-independence in this strain. Eventually knowing the properties of this strain and using various isogenic mutants allowed me to precisely titer choline content and study the impact of this aminoalcohol on severe pneumococcal diseases, such as sepsis and meningitis. Next I analyzed the differing immune responses induced by wildtype and mutant strains. My results actually indicated that *S. pneumoniae* attaches choline as a protection mechanism against clearance by the immune system. In the final vaccination studies of this work I obtained the promising results that choline-free strains may have the potential to be used as live-attenuated vaccine strains to protect against subsequent infections with a parental wild type strain.

3.2.1 Mechanism of Choline-independence in Strain R6Cho$^-$

In wildtype strain R6 the choline utilization genes are organized in two genetic loci on the chromosome: the *lic1*and *lic2* operons. R6Cho$^-$ was the result of a transformation of R6 with genomic *S. oralis* DNA. This transformation process equipped the resultant mutant R6Cho$^-$ with the ability to grow without choline. A loss of a Sma I restriction site in gene *spr1226* was discovered in R6Cho$^-$, indicating a genetic rearrangement in this genetic loci [41]. To fully understand the choline-independence in this strain, previous studies started to investigate the known wildtype genes first.

For instance, the existence of an active *lic1* operon in R6Cho$^-$ [41] was demonstrated by detection of a polycistronic mRNA containing the transcripts of the five genes *tarI/J, licA, licB* and *licC* [41]. These genes of the *lic1* operon were shown to be essential for the utilization of choline and for growth and survival of all isolates of *S. pneumoniae*. In contrast, R6Cho$^-$ with inactivated *licA, licB* or *licC* continued to grow

in choline containing growth media in the form of long autolysis resistant chains, indicating that these wildtype *lic1* genes are neither essential for viability nor for the choline-independence of the Cho$^-$ background [41].

In the course of my studies the mechanism of choline-independence in mutant R6Chi was discovered and assigned to a G→T point mutation at base position 700 in *tacF,* the first gene in the *lic2* operon [28]. It is assumed that bacteria expressing the wildtype TacF flippase can only transfer cholinated teichoic acid precursors to their surface. This structural requirement appears to be lost in the *tacF* G700T point mutant which can also transport choline-free teichoic acid chains and thus provides strain R6Chi with a choline-independent phenotype [28].

Strain R6Cho$^-$ seems to have retained the wildtype TacF which only transfers cholinated TAs. This was confirmed by sequencing the *tacF* gene of R6Cho$^-$ [27]. Consequently, the survival and physiological properties of a R6Cho$^-$ derivative from which the entire *lic2* operon (*tacF*, *licD1*, *licD2*) was deleted clearly indicate that genes of the *lic2* operon play no roles in the choline independent phenotype of this strain. Despite the lack of the two parental phopshorylcholine transferases LicD1 and LicD2, the mutant strain was still able to incorporate choline into its cell wall, although choline content was reduced to about 50%. Therefore, one could predict from these experiments that the acquisition of genetic elements from the heterologous *S. oralis* strain had to include not only a functional equivalent of *tacF* but also one phosphorylcholine transferase replacing the function of the pneumococcal *licD1* or *licD2*. The nucleotide sequence of the *S. oralis* insertion in R6Cho$^-$ is fully consistent with this prediction as it revealed homologues of *tacF* and *licD* (see Figure 15B).

The most likely candidate to carry out the phosphorylcholine transferase reaction in strain Cho$^-$ is the protein encoded by the imported *S. oralis* gene *licD4.* Its C-terminal domain is most similar to the LicD1 and LicD2 proteins of *S. pneumoniae* showing 35 % and 32 % identical residues, respectively. Another LicD domain is found in the gene product of *orf4* but similarity is rather limited. The LicD3 protein is also less likely to be involved in phosphorylcholine transfer since the very similar pneumococcal LicD3 (identity of 93 %) plays no role in this process. This is confirmed by another finding that strain D39ChiΔ*licD1D2* is free of surface bound choline despite the fully

functional *licD3* gene [27]. Gene inactivation studies will be needed to unambiguously identify the phosphorylcholine transferase(s) encoded in this region.

The second component expected to be present in the heterologous *S. oralis* DNA is *tacF*, encoding a teichoic acid flippase. While the endogenous R6 gene is part of the *lic2* operon located about 60 kb apart from the *licD3* locus, *tacF* of *S. oralis* is located immediately downstream of the presumptive *licD3* operon (see Figure 15B) and appears to be a single transcriptional unit. In contrast to TacF of R6, the acquired *S. oralis* flippase is able to export teichoic acids with or without attached phosphorylcholine groups as indicated by the ability of *S. oralis* to grow in choline containing and choline free medium as well [125].

In addition to *tacF* and *licD* genes, a gene (*orf2*) for a glycosyl transferase, a small gene (*orf5*) encoding a protein of unknown function, and *pce* specifying phosphorylcholine esterase [29] are also present in the acquired *S. oralis* DNA. Interestingly, the glycosyl transferase of *orf2* is similar (42 % identical aa) to that of *spr1223* which was lost upon the *S. oralis* DNA insertion (see Figure 15B). Since the wildtype *pce* of R6 is located outside of the *licD3* region, R6Cho$^-$ is equipped with two phosphorylcholine esterases.

The choline-independent mutant R6Cho$^-$ shows physiological abnormalities of chain formation and defective autolysis when grown in choline-free media. These phenotypes are understandable in terms of the known structural requirements of two pneumococcal enzymes (LytB and LytA) both of which require choline-residues in the cell wall for their activity [34,35,126]. As seen in the *lic2* deletion mutants of R6Cho$^-$, I found that 50 % of surface-bound choline was still sufficient to guarantee a functioning autolytic system, to allow complete daughter cell separation and to assure the wildtype diplococcal morphology of the bacteria.

Interestingly, these data also shed light on another important aspect of choline auxotrophy of wildtype *S. pneumoniae*. Interpreting these findings, the limiting factor on growth or survival is not necessarily choline itself, but rather the blocked transfer of intracellular, uncholinated teichoic acid chains. However, it is not clear whether it is the lack of surface-bound teichoic acids or the cytosolic precursor accumulation and subsequent clogging of synthesis pathways that have detrimental effects on the bac-

terium. It has been suggested that transfer of peptidoglycan building blocks and teichoic acids chains to the cell surface may be catalysed by the same system [38].

3.2.2 The Role of Choline Residues in Meningitis and Sepsis

A previous report showed that the high degree of invasiveness of *S. pneumoniae* required the presence of choline residues on the pneumococcal surface [41].

It was interesting to test whether choline in the cell wall actually represents a universal virulence factor that is essential for the manifold pathologies observed in the various pneumococcal disease models. Therefore an isogenic pair D39Cho$^-$ and D39Cho$^-$licA64 was tested in both the infant rat model of meningitis and the murine sepsis model. Strain D39Cho$^-$licA64 was free of choline, since a mutation in *licA* blocked utilization of choline from the *in vivo* environment.

Inoculated into the cisterna magna, the choline containing D39Cho$^-$ established a persistent infection in the CSF with detrimental effects on the host. The sequelae were severe meningitis, cortical and hippocampal damage and death of the animals (see Figures 17,18,19). Similarily, intraperitoneal application of the same strain led to bacteraemia and lethal sepsis in mice. In sharp contrast, the isogenic choline-free strain D39Cho$^-$licA64 showed radically reduced virulence approaching that of a capsule-free strain (see Figures 16A, 23A).

In either disease models the viable titers of both the choline-containing strain D39Cho$^-$ and the choline-free D39Cho$^-$licA64 increased substantially during the initial phase of the infection (see Figures 16B, 23B). Simultaneously, the immune response was activated and the proinflammatory cytokines IL-1β, IL-6, IL-12(p70) and TNF-α were expressed (see Figures 20, 25). Past a certain time point the activated innate immune response successfully contained the growth of choline-free bacteria. Declining bacterial blood titers were paralleled by decreasing cytokine levels. In contrast, choline-containing pneumococci continued to multiply despite the onset of an immune response. The ongoing bacterial proliferation led to steadily increasing levels of proinflammatory cytokines. The ultimate death of the animals might be a consequence of this overwhelming and persistent immune response. In particular TNF-α is a well-known inducer of lethal septic shock. Interestingly the expression of the anti-

inflammatory IL-10 during meningitis might be a futile attempt of the immune system to terminate the pathological inflammation. In general it is apparent that both bacteria triggered an immune response of comparable intensity during the onset of infection. However, an ongoing, late inflammation can only be observed and associated with the choline-containing, virulent bacteria.

From these results one might speculate that the choline residues are a pathogen-associated molecular pattern (PAMPs) that is directly recognized by PRRs of the immune cells. Therefore their presence could stimulate cytokine production and the observed pathological immune response. There are three types of receptors that may detect choline residues: Toll-like receptor 2 (TLR-2), rPaf and C-reactive protein.

First, I considered the possibility that the choline residues are directly detected by the TLR-2. A recent publication suggested that pneumococcal LTA binds by Toll-like receptor 2 *in vitro*, resulting in the expression of the cytokines IL-1, IL-8, IL-10, TNF-α, IFN-γ [127]. This pathway could contribute to the observed overwhelming immune response induced by choline containing bacteria as well as to their lethality. In turn, the lack of covalently attached phosphorylcholine residues may weaken the overall binding of LTA to TLR-2 and impair the virulence of the bacteria. If these assumptions were true, TLR-2 knock-out mice should be less susceptible to choline-containing bacteria. This hypothesis was tested by infecting TLR-2 knockout mice with D39Cho$^-$. Mortality was identical to what was observed in the isogenic wildtype mice (see Figure 24), indicating that the pathway via TLR-2 has no influence on the recognition of the choline moieties and the choline-dependent virulence of the strain tested.

But TLR-2 is not the only immune receptor that could potentially detect choline residues. Choline also binds to the receptor of the Platelet-activating-factor (rPaf) [85]. Although partially contributing to the mortality during sepsis [128], the interaction between choline and the rPaf seems to be of minor relevancy for cytokine production since binding of pneumococcal choline to rPaf does not induce any signal transduction within the host cell [85]. Thus it is unlikely to be responsible for cytokine secretion, as confirmed by a publication using purified cell wall as stimulus for cytokines in an *in vitro* assay [128].

Another alternative for choline detection is the soluble, IL-6 induced C-reactive protein (CRP). CRP was originally discovered as a serum protein that specifically recognizes phosphorylcholine residues on the pneumococcal teichoic acid [60]. A recent publication showed that CRP-opsonation of *S. pneumoniae* strain R36A resulted in enhanced *in vitro* cytokine expression by peripheral blood mononuclear cells (PBMC) [58]. However, CRP has not been detected in the murine host yet.

Therefore it seems valid to conclude that choline is most likely not recognized by any of the three known choline-specific immune receptors.

This is actually confirmed by the results gained from inoculating purified cell walls into the cisterna magna (see Figure 22). Independent of the presence of choline residues, both choline containing and choline-free cell wall preparations were able to trigger production of comparable levels of cytokines in the CSF. This is in line with previous studies in which pneumococcal cell wall preparations were shown to induce elevated white blood cell counts in a rabbit meningitis model. In the same study, substitution of choline residues by ethanolamine in the cell wall teichoic acid did not alter the efficacy of the cell wall preparations [129]. Furthermore, purified peptidoglycan (free of teichoic acid) was also shown to induce sepsis-associated neuronal damage in the dentate gyrus of mice [130]. Therefore my results suggest that the immune response was induced by a pneumococcal cell wall component that was common to both choline-free and choline-containing bacteria. Choline residues and their recognition by TLR-2, Paf receptor and CRP are not needed for the activation of the immune system.

To confirm that both bacteria indeed possess comparable immunogenicity, bacterial preparations were used to stimulate *in vitro* maturation of human monocyte-derived dendritic cells (see Figure 27). Human dendritic cells were incubated with Mitomycin C killed suspensions of strains D39Cho$^-$licA64 and D39Cho$^-$. In both cases DCs upregulated costimulatory molecules, although the choline-containing strain D39Cho$^-$ led to a slightly stronger activation. To narrow down the immunogenic epitope of the bacterial surface, purified cell walls differing in the presence or absence of choline, were also tested in the same assay and were found to have equal potency to induce maturation of dendritic cells.

To see whether these observations are also true in the mouse model of sepsis, I compared the maturation status of dendritic cells in CD1 mice that received the choline-free and choline-containing bacteria. Spleens were removed from mice and costimulatory molecules on the surface of isolated splenic dendritic cells were determined as indicators of maturation. Remarkably, both the choline-free as well as choline-containing strains equally engaged dendritic cells, despite the different intensities of the induced cytokine patterns. Lymphoid $CD11c^{+}CD8^{+}$ as well as myeloid $CD11c^{+}CD8^{-}$ DC subsets showed comparable upregulation of costimulatory molecules CD80 and CD86 (see Figure 26).

Taken together, these *in vivo* and *in vitro* experiments reveal a very important aspect of the pathogenicity of *S. pneumoniae*. In spite of their differences in the presence of choline, both bacteria possess the same immunogenicity. This means that the choline residue is not an immunogenic epitope. Therefore the powerful effect of choline residues on pneumococcal virulence has to be of a different nature.

It is conceivable that the very low invasiveness of choline-free pneumococci is an indirect consequence of the autolysis negative phenotype of the bacteria since the lack of choline residues in the cell wall protects pneumococci against the hydrolytic action of LytA. This amidase is needed for the release of the intracellular toxin pneumolysin from the bacteria [129]. However, autolysis and the release of pneumolysin is unlikely to play a critical role during the initial phase of the infection since both strains stimulated a similar initial immune response. On the other hand, since pneumolysin is a known TLR-4 ligand [72], its release might contribute to the late immune response only observed in animals infected with choline containing (autolysis-prone) bacteria. In general, the effect of LytA and pneumolysin on virulence is still controversial, since studies of autolysin-deficient mutants in sepsis models led to opposing results [99,100,101].

Therefore another explanation for choline-dependent virulence seemed to be more reasonable. Both bacteria were shown to have similar growth rates [41] and the same inflammatory potential. Therefore the most important difference between choline containing (highly virulent) and choline-free (virtually avirulent) pneumococci appears to be the differential clearance of these strains from the CSF or bloodstream. During meningitis the choline containing strain persisted and continued to grow in the CSF

even in the presence of an intense inflammatory response with a strong granulocytic pleiocytosis. In contrast, the choline-free organism which initially also grew and induced inflammation was eventually eliminated from the CSF with a rate that paralleled the appearance of a significant WBC response peaking at around 8 h after infection (see Figure 21). A similar correlation between choline-dependent bacterial growth and degree of inflammation was also observed in the murine sepsis model.

Combining these findings allows two major conclusions:

(1) Despite its drastically reduced virulence, the choline-free double mutant was still able to engage the host immune system as evidenced by the expression of proinflammatory cytokines, maturation of dendritic cells and the capacity to induce the influx of neutrophils into the CSF. The chemical entity responsible for triggering the observed immune response appears to be a component common to choline-containing and choline-free cell walls, most likely a constituent of the peptidoglycan or teichoic acid backbone.

(2) The striking difference in the virulence of the choline containing and choline free bacteria is most likely related to the superior capacity of choline containing cells to continue proliferation and reach higher titers in the host thus providing a continuous stimulus for cytokine production and keeping the immune system in a highly activated state for a prolonged time. In sharp contrast, the clearance of the choline-free bacteria from the system and their decreasing titers limit the pathological inflammation.

The significance of choline during infection is further emphasized if one considers that *S. pneumoniae* exists in two different growth modalities, depending on the infection site: a planktonic appearance during sepsis and a biofilm mode of growth in meningitis. These two states are characterized by a very different gene expression pattern which involves differential transcription of 10 prominent virulence genes [82]. Therefore choline in the cell wall of *S. pneumoniae* emerges as a newly recognized virulence factor of pivotal importance. Remarkably and in contrast to other described virulence factors, the essentiality of choline for causing lethal disease in the host appears to be maintained between different growth modalities.

3.2.3 The Role of Choline Residues in Immune Clearance of *S. pneumoniae*

In the previous part of the thesis I demonstrated that the critical contribution of choline residues to the virulence potential of pneumococci appears to be the role that these amino alcohol residues play in a pneumococcal immune evasion strategy. This evasion strategy protects the bacteria from the host immune system and allows their growth within the bloodstream. In this subsequent part of the thesis I aimed to test whether the observed clearance of choline-free bacteria from the bloodstream is due to an intrinsic factor of murine serum.

Mice were infected with choline free D39Cho$^-$licA64 and bacterial serum titers were monitored over time (see Figure 28A). After an initial growth a drop in viable titers was observed between the sixth and the ninth hour post infection [41]. Serum was collected at the 9 h p.i., assuming bactericidal levels of the hypothetical serum factor at this timepoint.

In contrast to the cholinated strain D39Cho$^-$, *ex vivo* reinoculation of isolated serum with D39Cho$^-$licA64 showed that choline-free bacteria completely lost the ability to grow and were cleared by some antimicrobial activity of the serum (see Figure 28B). A conceivable explanation for this striking observation could be that choline-free bacteria were highly susceptible to a well-known antimicrobial serum component: the complement system. However complement mediated killing in this *ex vivo* setting is very unlikely, since pneumococci are protected by their thick peptidoglycan layer from the deposition of the bactericidal membrane-attacking complex C5b-9 [57]. Moreover it was shown that the pneumococcal capsule inhibits the deposition of C3b on the bacterial surface [102]. Additionally, *S. pneumoniae* produces a surface protein called Factor H-binding inhibitor of complement (Hic) that recruits and attaches the host's complement regulator Factor H to the pneumococcal surface thus preventing the binding of C3b as well [103]. The latter two virulence mechanisms of pneumococci would reduce complement-mediated opsonophagocytosis of the bacteria. Despite these facts, I sought to remove this variable, by heat inactivating and sterilizing the serum (hi-serum), thus destroying the complement system [131] and any unlikely eukaryotic phagocytic cell contaminant. Heat-inactivated serum still allowed growth of cholinated and clearance of choline-free bacteria (see Figure 28C).

If the observed pneumococcal evasion mechanism against the serum clearance is really choline-mediated, the blocking of the choline-residues of D39Cho$^-$ should impair growth in serum. To manipulate choline content of D39Cho$^-$ I used two immune molecules IgA TEPC-15 and C-reactive protein (CRP) that are specific to the choline residues of the (lipo)teichoic acid [26,60]. By this I was able not only to have a way to shield all the vacant, unbound choline residues on the bacterial surface, but also to better mimic the *in vivo* situation, in which both of these proteins were shown to be abundantly present during pneumococcal infections [132,133]. Since CRP is a very effective activator of complement [58] experiments were conducted in complement-free, hi-serum. And in fact, the presence of IgA TEPC-15 or CRP drastically decreased serum-resistance of the cholinated strain D39Cho$^-$ (see Figure 28D).

Excluding complement, the clearance of pneumococci from the serum *ex vivo* can be attributed to a non-cellular, heat-stable serum factor. Further, bacteria are protected from this antimicrobial serum activity by choline-residues on their surface, an evasion mechanism that can be abolished by neutralizing these aminoalcohols with the host immune molecules IgA TEPC-15 and CRP.

To understand whether the demonstrated IgA TEPC-15/CRP-amplified serum factor killing plays a role during sepsis *in vivo*, mice were passively immunized with these immune molecules prior to an intraperitoneal inoculation with D39Cho$^-$. Both (IgA TEPC-15 and CRP) prolonged survival of the animals (see Figure 32), most likely due to enhanced bacterial clearance. In the case of CRP bacterial killing is probably only partially achieved by the heat-stable serum factor, since it was shown that protection of passively CRP-immunized mice against a serotype III strain Pn3 was also dependent on an intact complement system [65]. Although my *in vitro* findings showed that complement is not likely to influence killing by serum, it can enhance uptake by phagocytes in the animal host *in vivo*.

A better dissection of the *in vivo* killing process of the bacteria allows the IgA antibody, that barely induces complement [53]. In this case the partial protection of the animals is most likely mediated by antagonizing pneumococcal resistance towards the serum factor. However, studies using choline-specific IgG/IgM antibodies that could potentially trigger serum factor and complement mediated killing showed full systemic protection of mice from strain WU2 septicemia [134].

One might argue that the growth in long chains and the unknown fate of choline-binding proteins (CBPs) in the choline free strain D39Cho$^-$licA64 might be the actual reason for the observed loss of virulence in the various animal models. Similarly it is conceivable that the choline-specific IgA and CRP interfere with CBP expression and displace these proteins from the surface of D39Cho$^-$, thus introducing secondary artefacts, similar to the growth defects in the licA$^-$ mutant.

To exclude these potentially interfering factors, I investigated the growth dynamics and behavior of D39Cho$^-$ in medium in the presence of either TEPC-15 or CRP, at the respective concentrations that already had an inhibitory effect on growth in the *ex vivo* serum. Blocking of choline residues did neither impact growth rates nor diplococcal phenotypes, indicative of physiologically fully functioning bacteria with unaltered CBPs (see Figure 29A). To further confirm the presence of CBPs deoxycholate induced lysis of the cultures was tested. Lysis is inhibited in mutants lacking functional autolysin LytA. One such mutant strain is D39Cho$^-$licA64 which served as negative control in this assay and did not lyse. In contrast, cells previously treated with TEPC-15 or CRP were still prone to lysis and no difference to untreated D39Cho$^-$ was observed (see Figure 28B). Therefore we conclude that TEPC-15 and CRP only bind to vacant choline-residues and do not compete with the attachment of CBPs so that all choline-associated physiological features of the bacteria are maintained and function properly. Accordingly the clearance of TEPC-15/CRP-treated D39Cho$^-$ from serum is not due to a growth impairment of the bacteria.

Interestingly, the results from the infant rat model of meningitis demonstrated a similar choline-associated clearance pattern of choline-free bacteria from the cerebrospinal fluid (CSF). Although these two infection sites (serum, CSF) represent immunological compartments that are very distinct in their predominant immune effector mechanisms, the expression of heat-stable, cationic antimicrobial peptides (CAMPs) during bacterial sepsis or meningitis is common to all of them. A study in patients suffering from bacterial infections revealed that concentrations of active human neutrophil peptides 1-3 (HNP 1-3) were significantly elevated in blood, plasma, pleural fluid and CSF [48]. Another publication demonstrated that defensins were upregulated in children with bacterial meningitis [49]. In the mouse model of meningitis the expression of CRAMP was demonstrated as a response to an infection with *Neisseria men-*

ingitidis [47]. Consistently, the respective human and rat homologues LL-37 and rCRAMP can be found in CSF samples collected during bacterial meningitis, also induced by *S. pneumoniae* [51].

To understand if these CAMPs could be the suspected heat-stable serum component, I tested the antibacterial effect of the lantibiotic Nisin - one of the most intensively studied CAMP [135] - on the killing of *S. pneumoniae in vitro*. Nisin was also described to protect mice from pneumococcal bacteraemia *in vivo* upon passive administration [136]. Identical to the bacterial killing by *ex vivo* serum, cholination of (lipo)teichoic acids in wildtype strains is linked to resistance against Nisin (see Figure 31A,B). In sharp contrast, the isogenic choline-free *licA*-depleted derivates were prone to the antimicrobial activity of Nisin. Interestingly, Nisin even induced killing of the choline-free strain D39Cho$^-$licA64 independent of lysis and an autolytic system. Consistently with the observations in the *ex vivo* serum, treatment of D39Cho$^-$ with TEPC-15 and CRP resulted in a drastic loss of resistance towards Nisin, increased lysis (see Figure 31A) and a drop in viable counts, comparable to those of the choline-free D39Cho$^-$licA64 (see Figure 31B).

Taken together, serum and CAMP resistance is exclusively linked to the choline residues of the (lipo)teichoic acids and specific blocking of these amino-alcohols by IgA or CRP host molecules can counteract with this resistance mechanism of *S. pneumoniae*.

But how can a choline-dependent resistance mechanism be explained? What is the role of choline-specific IgA or CRP? First of all, the abundant incorporation of choline residues into the cell membrane is a feature common to all eukaryotic cells and is believed to protect the host against the lethal properties of secreted CAMPs. Therefore the decoration of the pneumococcal surface may be interpreted as a molecular mimicry of *S. pneumoniae*, imitating host cell surfaces. The mode of action of antimicrobial peptides is mainly mediated through their cationic charge, which attracts them towards the anionic phospholipids of bacterial membranes. Due to their hydrophobicity the peptides are then able to penetrate the lipid bilayer, form pores and eventually kill the pathogen [52].

Choline is a positively charged aminoalcohol that, by attachment to the bacterial cell wall, is able to reduce the overall net negative charge of the bacterial surface and to

repel the peptides in a charge - dependent manner. It was already demonstrated that covalent linkage of D-alanyl esters to teichoic acids in strain D39 works in a similar fashion and decreases the susceptibility to CAMPs of *S. pneumoniae* [17]. Another publication described that choline-residues confer CAMP protection to the bacterium *Haemophilus influenzae*, a frequent colonizer of the human respiratory tract [137]. Interestingly, several microbial pathogens that aim to colonize the respiratory tract express choline on their surface [32]. In particular interesting is the fact that transparent colonies of *S. pneumoniae*, which are the pneumococcal growth phase during colonization, were shown to upregulate the expression of cholinated teichoic acids [81].

Enhanced choline presentation during mucosal colonization might be beneficial for the following reason: CAMPs are (together with IgA antibodies) very prominent innate immune mechanism of the mucosal compartment [50,53]. If my proposition of a choline-CAMP interaction was true, choline-residues should be even more important for colonization than for sepsis.

Indeed, blocking of vacant choline residues had a dramatic effect on the colonization capacities of the pneumococcus, so that only 5% of the IgA TEPC-15/CRP-treated bacteria could be recovered from the nasopharynx. Although choline is described as a ligand for the receptor of the Platelet-activating factor (rPaf) which promotes adherence to epithelial cells [85], it is unlikely that the loss of this pathway is the exclusive explanation for the drastic drop in colonization for several reasons. For example, the same study showed that the use of an rPaf antagonist only partially inhibits colonization [85]. Even more striking is the fact that *lic2* operon mutants still possess one choline residue per TA but already showed a reduction of colonizing capacity by 75% (see Figure 30). These very strains maintained wildtype phenotypes (see Table 2) and therefore should be able to utilize the full arsenal of choline-dependent adherence factors such as the rPaf pathway and CBP adhesion. In the same way, my *in vitro* growth curves (see Figure 29) showed that TEPC-15/CRP treated D39Cho$^-$ can still rely on functional CBPs, presumably also under *in vivo* conditions in the murine nasopharynx.

Generally, adhesion of *S. pneumoniae* to the nasopharynx is a redundant process and can be accomplished by several choline-independent pathways as well. There-

fore, even choline-free bacteria, can still adhere to Detroit 562 pharyngeal cells *in vitro* [41]. Also, the lipoprotein PsaA possesses adhesive properties. *S. pneumoniae* has also the ability to interact with glycoconjugates on host cells. The addition of N-acetylglucosamine-β-1-3-Galactose (Glc*N*Acβ1→3Galβ) blocks attachment to human pharyngeal cells *in vitro* [86]. Also, other oligosaccharides are involved in the adherence of pneumococci to epithelial cells [87,88,89].

Therefore the IgA/CRP-blocking of choline residue is likely to reduce D39Cho$^-$ colonization by a composite process that abolishes a pneumococcal immune evasion mechanism (most likely against CAMPs) on the one hand, and the choline-rPaf pathway on the other.

These findings might even have bearing on observations described in two publications about *H. influenza* which showed that (i) choline residues protect these bacteria against the antimicrobial peptide LL-37, secreted into the respiratory tract [137], and that (ii) CRP - expressed in the airway and nasal surface fluid - contributes to the clearance of *H. influenza* [59]. The authors of the latter study speculate about an unknown antimicrobial activity of CRP that depends on the presence of an additional undefined factor on the mucosal surface of the human respiratory tract [59]. Assuming that these unknown factors are cationic antimicrobial peptides and connecting the two observations with my results actually implies that a cooperative bacterial clearance mechanism by CRP and the CAMP LL-37 may have been observed before but not recognized as such. This hypothesis is even further supported by the finding that patients during bacteraemia had significantly elevated blood and plasma levels of active human neutrophil peptides 1-3 (HNP 1-3) together with an increased production of serum CRP concentrations [48].

In this last part of the thesis I found that the positively charged choline residues of the TA and LTA of *S. pneumoniae* protect the bacterial cell envelope from the charge-dependent antimicrobial action of purified CAMPs or murine serum, respectively. This immune evasion mechanism seems to play a key role in colonization and sepsis. Consistent with this hypothesis, a mutant strain with an inactivated phosphorylcholine-esterase Pce, (which carries more choline-residues than its parental strain) shows greatly enhanced virulence in the intraperitoneal mouse model [29]. I found

that *in vivo* blocking of this aminoalcohol by passive immunization with IgA TEPC-15 and CRP partially protected mice against sepsis and nasopharyngeal colonization.

Usually an infection of naïve mice with cholinated pneumococci leads to a fatal outcome of the disease. Apparently, the proliferation rate of fully cholinated bacteria is superior to the rate of production of CRP or choline-specific immunoglobulins in the naïve animal. But strengthening my hypothesis is the fact that if mice were vaccinated with phosphorylcholine and got time to develop pre-existing choline-specific antibodies they were protected against mucosal colonization by *S. pneumoniae* [138].

Taken together these findings allow one to consider a common, novel function of CRP and antibodies that might apply to a variety of other innate immune molecules as well. Neutralization of positively charged surface structures of pathogens can abolish resistance against serum *ex vivo* and the cationic antimicrobial peptide Nisin *in vitro*. In a similar fashion, antibodies could even be seen as a link between adaptive and innate immunity to target and amplify the usually unspecific immune response. This is of particular interest for IgA antibodies, the functions of which are still elusive. I propose that IgA antibodies might interact with cationic antimicrobial peptides during host defense. Both of these immune molecules are abundantly present on mucosal surfaces. Further, these results might be beneficial for the design of vaccines against all kinds of pathogens that colonize the mucosa: stimulating antibody responses against positively charged surface structures might enhance the protective potential of these vaccines.

3.2.4 Alternative Host Clearance Mechanisms and Future Projects

Clearance of the choline-free bacteria is most likely a process that is cooperatively achieved by several alternative immune effector mechanisms.

For example, the choline-free organisms may produce a less protective capsule while growing *in vivo*. Although the presence of capsular polysaccharides in choline-free bacteria was demonstrated [41], the amount was never quantitatively assessed. With a weaker capsule choline-free pneumococci may be more susceptible to other host defense factors (such as complement).

A weaker capsule production may occur if the expression of capsular polysaccharides and their amount on the pneumococcal surface is directly correlated to the presence of choline and/or the teichoic acid pathway. As already described in the introduction, the synthesis of these two polysaccharides (capsule and TA) is very much alike and includes common chemical precursor molecules (e.g. UDP, and other Nucleotid-sugar-precursors...). Since neither of the pathways is presently fully understood there may even be more overlapping molecules and or protein catalysts involved.

For instance it is conceivable that chemically different components of the cell surface are delivered through shared transport ligands, e.g. bactoprenol molecules that have to be released and recycled during teichoic acids synthesis to enable the subsequent transport of capsular polysaccharides or peptidoglycan chains. It seems that the crucial step in the recycling process of these precursor molecules is coupled to the activity of the TacF flippase and the transport of teichoic acids to the surface. Although the transport of choline-free teichoic acid chains can be achieved by the choline-independent mutant bacteria, the flipping catalyzed by the mutant TacF might occur at a lower rate. This may slow down polysaccharide-synthesis and lead to a bacterium with a weaker capsule. It needs to be mentioned that the presence of the capsule is not essential for the viability of the bacterium *in vitro*, which is demonstrated by a variety of unencapsulated, rough laboratory strains.

In contrast to the non-capsular polysaccharide synthesis pathway, which is not essential for growth *in vitro*, the very similar peptidoglycan synthesis pathway is essential for pneumococcal viability. Comparable to the synthesis of capsular polysaccharides and TA, this third pathway for peptidoglycan synthesis may also use this common carrier lipid molecule. Therefore it was speculated [139] that a block of the choline-dependent regeneration of the common precursor from the TA pathway in a wildtype bacterium, will also lead to a halt of peptidoglycan synthesis, which may be responsible for the lethal effects on the cell.

Future work could focus on the evaluation of this choline-dependent capsule expression. If the above mentioned theory was true, it would be interesting to test the virulence of other available choline-independent bacterial mutants that posses alternative tacF flippases (501, JY2190). Maybe the TA turnover kinetics of these tacF mutants

enable a faster precursor recycling, resulting in a more protective capsule and altered virulence. This would mean that different tacF alleles can be used as a tool for titering capsular expression. It would be very interesting to quantitatively asses the production of capsular polysaccharides in various choline-independent mutants. It is also possible that choline in the cell wall has a direct regulatory effect on synthesis of capsular polysaccharides. Northern Blots would reveal differences in *cps* locus transcription profiles with respect to choline availability in the growth medium.

Another important variable for bacterial clearance by the host could be the unknown status and/or localization of choline binding proteins (CBPs) in the choline-free mutant. Several members of this family of proteins were shown to be associated with virulence [29,140,141] or immune protective potential [142]. As a consequence of CBP deprivation the autolytic system is impaired and choline-free pneumococci grow in long chains. These chains may present a greatly increased target size for phagocytic cells of the host. However, mimicking chain formation of choline containing bacteria by mutating genes *lytA* and *lytB* did not result in such a drastic loss of virulence in a murine sepsis model, demonstrating that the avirulence of the choline-free bacteria cannot be exclusively linked to the growth in long chains [41]. It is rather possible that the loss of the choline residue and a change in the surface charge might also have an effect on phagocytosis. For instance, phagocytic scavenger receptors recognize negative charges. Binding studies between cholinated pneumococcal teichoic acids and scavenger receptor-I suggested that the positive choline charge actually interferes with the binding [67] and could counteract phagocytosis. This might explain the enhanced clearance of choline-free bacteria as well.

To understand the effect of choline in the cell wall on phagocytosis a variety of experiments could be done. Studies on splenectomized animals revealed that the spleen is of crucial importance for the removal of *S. pneumoniae* from the host. In a first setup the cemetery of choline-free bacteria should be determined by microscopically localizing labeled bacteria in the spleen. From the anatomy of the spleen one could infer which phagocytic cell type is mainly involved in the clearance. The use of knock-out mice, deficient in phagocytic receptors SIGN-R1 or MARCO might even give further information about the specific receptor-pathogen interactions. Subsequent *in vitro* phagocytosis assays could further narrow down the recognized bacte-

rial surface epitope. One could purify cholinated and unsubstituted WTAs and LTAs and coat latex beads with these components. Applying these coated beads on cell culture phagocytes (e.g. macrophages) would give an understanding whether choline-free TA are better ingested by the eukaryotic cells. A similar phagocytosis *in vitro* approach was already used for lipid components of mycobacteria [143].

3.2.5 Protective Potential of Choline-free Strains

Two major findings of this thesis can be summarized as follows. First, choline-free bacteria are attenuated because they lost an immune evasion mechanism that protects them against host clearance. Second, although avirulent, choline-free bacteria were immunogenic enough to engage the immune system. Therefore I was interested to test whether or not such a immunogenic, yet avirulent bacterium could be exploited as a live-attenuated vaccine to induce protection.

To explore this possibility, mice were immunized with a single dose of D39Cho$^-$licA64. After 10 days the same animals were challenged with the isogenic virulent strain D39Cho$^-$ and a surprisingly strong protection was detected. However, it was not clear from this experiment whether the induced protection was serotype-specific or may be related to the large heterologous *S. oralis* elements that were shown to be present in the strain D39Cho$^-$.

To address these questions D39ChilicB31, a recently isolated and genetically very well defined strain which only differs (besides the mutation in the *licB* gene) by a single point mutation [28] from the wildtype D39 (and is therefore free of any possibly interfering, heterologous genetic elements) was also tested in the protection experiment. Table 4 shows that animals surviving infection by the avirulent double mutant D39ChilicB31 were protected against challenge by the highly virulent, isogenic wildtype strain D39 but the same animals were still susceptible to challenge by the capsular type III strain SV36. These findings make it unlikely that the protection effect originally observed was specific for the strain or to the large heterologous DNA sequences carried in D39Cho$^-$licA64, the strain used in the original protection experiment. Instead, the results suggest that infection by the avirulent pneumococci was able to generate protective immunity in mice that was specific for the capsular type.

This was also confirmed by the detection of capsule specific IgM antibodies in the serum (see Figure 34).

Future experiments would be needed to analyze the specifity and nature of the underlying immunological memory. Therefore long-term vaccination and challenge trials are needed to further characterize the induction memory response and its durability. Capsule-specific serum antibody titers at various timepoints could be determined and would give additional understanding of the protection. Also the use of knock out mice that are deficient in Fc receptors could reveal the importance of the different antibody isotypes in the mediation of long-term protection.

3.2.6 Future development of live-attenuated vaccine strains

This work presented the ability of live attenuated strains of *S. pneumoniae* - D39Cho$^-$ licA64 and D39ChilicB31 - to generate capsule-specific protective immunity in a murine sepsis model. The *in vitro* data on human monocyte-derived dendritic cells raise the hope that the findings might be extendable to the human host. Theoretically, this approach could be applied to a variety of serotypes representing a simple, yet powerful tool to produce customized vaccines according to the needs of a certain population and its prevalent serotypes.

The commonly used pneumococcal vaccines are based on the administration of a selection of either purified or conjugated (to carrier proteins) capsular polysaccharides. The two tested choline-free strains could be used in a similar approach as live attenuated bacteria representing a vehicle to deliver capsular polysaccharides. A big advantage is that the simple attenuation of assumingly all existing serotypes is a quick and easy two step process, consisting of two consecutive transformations. *S. pneumoniae* requires two operons for the utilization of choline: lic1 operon for the uptake of choline and lic2 operon for its attachment to the pneumococcal surface. In a first step one has to free the pneumococcus from its auxotrophy for choline by equipping the bacterium with either the Cho$^-$ allele or a point-mutated *tacF* gene and subsequently selecting for clones in a choline-free environment. In a second transformation the obtained choline-independent mutant has to be disabled to revert to a choline-containing phenotype in the presence of exogenous choline, which can be

achieved by inactivating either one of the genes licA, licB, or licC located in the lic1 operon [41].

Like with almost every other live attenuated vaccine there is always a possibility of spontaneous revertants that might regain full virulence. This concern might especially apply to *S. pneumoniae* which features naturally occurring competence rendering the pneumococcus into an unusual, genetically dynamic bacterium. However, since pneumococcal competence is dependent on the presence of surface-bound choline [31], the genetic flexibility of the live attenuated bacteria is strongly impaired, decreasing dramatically the probability of a reversion.

The absence of choline molecules from the surface would also assure that the intracellular toxin pneumolysin would not be released into the host because the enzyme LytA responsible for the release of pneumolysin could not function in the absence of choline residues.

The ultimate goal would be to develop a multivalent vaccine that allows easy application. To achieve that, one should test whether the simultaneous i.p. administration of multiple attenuated serotypes will establish systemic protection against the respective virulent parental strains. Secondly, an alternative, more practical route of application would be of use. The administration of live-attenuated strains into the nasopharyngeal compartment would be ideal. In case that the impact of the vaccine (intraperitoneal or nasopharyngeal application) has to be enhanced a secondary booster immunization could strengthen the protection.

3.3 Conclusion

The pneumococcal surface is the stage on which the bacterium interacts with the immune system of the host in the course of an infection. In gram-positive bacteria such as *S. pneumoniae* it is in particular the thick cell wall layer that is mainly exposed and a major target of the immune system. In this thesis I was able to show that two simple chemical modifications of this cell wall have profound effects on the fate and survival of not only the bacteria but also the host. Not being able to acetylate its peptidoglycan blocks the bacterium from colonizing the host. Even more dramatic

was the impact of removal of choline residues from the bacterial cell wall, which led to the complete loss of virulence and the survival of the host.

Further analysis of these findings suggested two strategies, which could contribute to an improved control of *S. pneumoniae* in the future. One was to specifically target and neutralize positively charged surface structures (e.g. choline). This could be achieved by either passive application of heterologous blocking reagents (human CRP in murine host) as performed in this work or by establishing a specific immunological memory response against these charged structures. The second, even more powerful strategy presented in this thesis was the promising use of an avirulent bacterium, which has the potential to be developed into a live attenuated vaccine strain.

Taken together, the findings of this thesis can be seen as a possible foundation for upcoming projects that aim to design immunization or treatment strategies against infections with *S. pneumoniae* or other mucosal, cholinated pathogens.

4 Material and Methods

4.1 Microbiological Methods

4.1.1 Cultivation of *S. pneumoniae*

All bacteria were cultured in liquid culture in either C+Y, Cden or Cden medium supplemented with 5µg/ml choline, depending on the experiment. An overview of the used strains is displayed in Table 7.

For infection studies in animal models investigating the role of choline residues in the disease, *S. pneumoniae* strains were grown in choline-free Cden medium at 37°C. By using choline-free Cden medium all strains showed a chain-forming phenotype just prior to infection of the animals. Choline auxotroph strains were grown in C+Y medium at 37°C. For inoculum preparation exponentially growing cultures of the strains were back diluted in the respective growth medium and were allowed to grow to OD_{590nm}=0.6. Bacterial pellets were washed twice with 0.9% NaCl. The cultures were further diluted in 0.9% NaCl and adjusted to the desired inoculum concentrations.

For enumeration of colony-forming units (CFU), serial dilutions of bacteria were plated on blood agar plates (BAP), consisting of Tryptic Soy Agar (TSA) supplemented with 5 µg/ml gentamicin and 3% sheep blood. Bacteria were incubated at 37°C under a 5% CO_2 atmosphere.

4.1.2 Deoxycholate-induced lysis of *S. pneumoniae*

For Deoxycholate lysis, 1 ml of bacterial culture at OD_{590nm}=0.7 was centrifuged and pellets were resuspended in 950 µl Phosphate-buffered saline (PBS, pH 7.2). Lysis was induced by addition of 50 µl Deoxycholate solution (4%) and optical densities were determined.

4.2Cell wall Purification and Analysis

4.2.1 Purification of Cell walls

Pneumococcal cell walls were prepared by a previously published method [144,145,146]. Bacteria were grown in 1 l of medium until OD_{590nm}=0.6 and incubated on ice for 5 min. Cells were harvested and resuspended in 40 ml icecold Tris/HCl 50 mM pH 7,0. Add the resuspended cells dropwise under stirring to 120 ml boiling SDS 5%, Tris/HCl 50 mM pH 7,0 and boil for 30 min. To remove the SDS the crude cell wall extracts were washed twice with 1 M NaCl followed by 10 wash steps with MilliQ water. The Hayashi test was used to detect traces of SDS. Therefore 335 µl of the wash supernatant were mixed with 170 µl Sodiumphosphat 700 mM (pH 7,2), 7 µl Methylene Blue 0,5% and 1 ml Chloroform. After brief vortexing for 30 sec, the lower chloroform phase should be transparent if no SDS is left anymore.

The SDS free pellet was resuspend in 3 ml MilliQ water and glass beads (170-180 µm diameter) were added. After breaking the cell walls with a Fast Prep 120, the glass beads were removed by filtration and the flowthrough was resuspended in 25 ml MilliQ water. Big debris was removed by gentle centrifugation and the supernatant containing the cell walls was collected and centrifuged. The pellet was resuspended in 2 ml 100 mM Tris/HCl (pH 7,5), 200 µl 200 mM $MgSO_4$, 10 µg/ml Dnase, 50 µg/ml Rnase and incubated for 2 h at 37°C. After that 10 mM $CaCl_2$ and Trypsin 100 µg/ml was added and incubated for 18 h at 37°C. 600 µl SDS 5% and 50 mM Tris/HCl (pH 7,0) and 20 ml MilliQ water were added. After centrifugation, the pellet was resuspended in 8 M LiCl. Following another centrifugation the pellet was resuspended in EDTA 100 mM (pH 7,0). After washing with MilliQ water and Acetone the cell walls were lyophilized and stored at -20°C.

4.2.2 Preparation and Analysis of the Cell wall Stem Peptides

Cell wall material (2 mg) was treated with affinity-purified pneumococcal amidase (5 µg). The solubilized cell wall material washed with acetone, and the peptides extracted with acetonitrile-isopropanol-water (25:25:50) containing 0.1% trifluoroacetic acid. After removal of the solvents by evaporation in a Speed Vac, the peptides were

dissolved in 0.1% trifluoroacetic acid. Peptides were separated with a Shimadzu LC-10AVP HPLC system on a Vydac 218TP54 column (W. R. Grace & Co.-Conn, Columbia, MD). The peptides were eluted with an 80 min linear gradient from 0% to 15% acetonitrile (Fisher Scientific, Pittsburgh, PA) in 0.1% trifluoroacetic acid (Pierce Biotechnology Inc., Rockford, IL) pumped at a flow rate of 0.5 ml/min. The eluted fractions were detected and quantified by determination of their UV absorption at 210 nm (A210).

4.2.3 Preparation and Analysis of Cell wall Muropeptides

Cell walls were prepared and analyzed according to a published procedure for preservation of *O*-linked acetyl groups [105]. Cell wall (1 mg) was treated with 20 μg of cellosyl (kindly provided by Hoechst, Frankfurt, Germany) in 20 mM sodium phosphate pH 6.0 at 37°C for 36 h. The samples were boiled for 5 min and centrifuged. The supernatant was reduced for 20 min at 20°C with 5 mg/ml of sodium borohydride in 0.5 M sodium borate buffer pH 8.0. Then the pH of the sample was adjusted to 5-6 with 20% phosphoric acid. Samples were separated by HPLC using a Prontosil 120-3-C18-AQ column (Bischoff, Leonberg, Germany) as described [105].

4.2.4 Mass Spectrometry of Muropeptides

The samples (HPLC fractions) were concentrated and desalted with the help of Zip-Tips (U-C18) from Millipore. Mass spectrometry was performed applying enhanced MS modes on a 4000 Q Trap mass spectrometer (Applied Biosystems). Nanospray (1200 V) was used for ionization, and masses in the range of 400 to 1100 *amu* were monitored.

4.2.5 Detection of alkaline-labile Acetate in Pneumococcal Cell wall

Cell wall was prepared from 2.5 l of pneumococcal cultures (in C+Y medium) with an optical density of about 0.6. The cells were harvested by centrifugation and washed twice with 40 ml of 0.9% sodium chloride. The pellet was re-suspended in 40 ml of

0.9% sodium chloride and the sample was boiled for 20 min. After chilling on ice for 5 min, the sample was centrifuged and the pellet re-suspended in 10 ml of 0.9% sodium chloride. The cells were broken with glass beads in a FastPrep FP120 disintegrator. After removal of the glass beads by filtration, the sample was centrifuged and re-suspended in 40 ml of water. The sample was next added to 120 ml of 5% SDS, 50 mM Tris/HCl, pH 7.0, and boiled for 30 min. After centrifugation, the pellet was re-suspended in 2 ml of 0.1 M Tris/HCl, pH 6.8. For the removal of proteins, 1 mg of Trypsin was added and the sample was stirred at 37°C for 16 h. The cell wall was collected by centrifugation, washed twice with 40 ml of water, and was lyophilized. Acetate was released from cell wall with 80 mM sodium hydroxide and was analyzed by HPLC as described [105].

4.2.6 Choline Content of purified Cell walls

To determine choline incorporation into the cell wall, strains were grown in Cden medium supplemented with 80 μCi and 5 μg/ml of ^{3}H-Choline to an OD_{590nm}=0.6. Cell walls were purified [38] and 100 μg purified cell walls were digested with muramidase and processed for quantification of radioactive counts.

4.3 The infant Rat Model of Meningitis

4.3.1 The Animal Model

The animal studies were approved by the Animal Care and Experimentation Committee of the Canton of Bern, Switzerland, and followed National Institutes of Health guidelines for the performance of animal experiments.

An established model of experimental pneumococcal meningitis in infant rats was used [112,147,148]. For the study with live bacteria two independent experiments were performed. In each of these experiments 11 day old infant Wistar rats (Charles River, Germany) were used. Animals were intracisternally (i.c.) infected with 10 μl of the prepared inocula. Group sizes of 6 animals (n=6) for the lower inoculum of 5 x 10^2 CFU and 8 animals (n=8) for the higher inoculum of 2 x 10^3 CFU were used per bacterial strain. At defined time intervals (4, 8, 12, 18, 24 and 36 h) the impact of

bacterial inocula on the disease severity of the animals was determined in the following manner. Animals were turned on their backs and the length of time needed for the animals to turn to their normal upright position was tested and scored by numbers: 5 (normal), 4 (turns upright in less than 5 seconds), 3 (turns upright in less than 30 seconds), 2 (does not turn upright), 1 (coma / death). Animals showing a score of 1-2 had to be sacrificed for ethical reasons (termination criteria). At each timepoint cerebrospinal fluid (CSF) was collected by puncturing the cisterna magna using a 32 gauge needle. Since animals needed time to recover from tapping they were divided into two equal groups, and cisternal puncture was alternated between the groups at different timepoints. For bacterial titer determination serial dilutions of 5 µl CSF were plated on 3 % sheep blood agar plates and incubated at 37°C in a 5 % CO_2 atmosphere. Remaining CSF was frozen on dry ice and stored at -80°C. For cytokine detection, CSF samples were centrifuged (15000 g, 10 min, 4°C) and supernatants were kept at -80°C. Survival of the rats was monitored over time and analyzed for significant difference performing a Logrank test. At the endpoints of the experiments and at timepoints of death brains were processed for histopathological analysis. Animals were perfused with 30 ml of ice-cold PBS via the left cardiac ventricle. The right hemisphere was fixed in 4 % Formaldehyde / PBS (pH 7.4) at 4°C for evaluation of brain damage in cortex and hippocampus by histomorphometry [109,147]. Animals that died unobserved were excluded from histopathological examinations.

4.3.2 Inoculation of Cell Walls into the CSF space

Choline-free and choline containing cell walls were prepared from the non-encapsulated strain R6Cho$^-$ after growing the bacteria in Cden medium free of choline or supplemented with choline. Suspensions of cell walls were adjusted to the desired concentration in 0.9 % NaCl and 10 µg cell walls (in 10 µl) corresponding to approximately 10^7 CFU equivalents were injected i.c. and CSF of 3 rats per timepoint was tapped and processed for cytokine expression and MPO activity as described below.

4.3.3 Cytokine Expression in the CSF

Microsphere-based multiplex assays (Lincoplex®, Linco Research Inc., St Charles, MA, USA) were used to assess the CSF concentrations of the following cytokines: IL-1β, IL-6, IL-10, IL-12(p70) and TNF-α. All CSF samples were diluted five fold to a final volume of 25 µl. A minimum of 100 beads per analyte was measured. Calibration curves from the recombinant standards provided were calculated with Bio-Plex Manager software version 4.1 using a five parametric logistic curve fitting. When cytokine concentration was below the detection limit an arbitrary value corresponding to five times the detection limit of undiluted samples was used for statistical analysis.

4.3.4 Myeloperoxidase Assay

5 µl of uncentrifuged CSF were resuspended in 200 µl HETAB solution (0.5% hexadecyltrimethylammonium bromide in 100 mM potassium phosphate buffer, pH 6.0), repetitively submitted to 3 cycles of freeze-thawing, sonicated and centrifuged for 5 min at 10000 g, 4ºC. Supernatants were stored at -80ºC until use. Assays were performed in triplicate, by mixing 155 µl of HETAB buffer with 10 µl of samples and 10 µl of o-dianisidine (20 mg/ml in water) in a 96 well plate. Reaction was initiated by the addition of 25 µl of 2 mM hydrogen peroxide in water. Absorbance was measured at 450 nm each 30 seconds for 10 min at 37ºC. The linear domain of the curve was used to determine the increase in absorbance. Myeloperoxidase (MPO) activity was calculated from a standard curve created with purified MPO from human leukocytes (Sigma), and expressed as mU/ml.

4.3.5 Matrix Metalloproteinase (MMP) Zymography

The amount of the constitutively expressed MMP-2 and the inducible MMP-9 in the CSF was measured by zymography using gelatin-containing gel electrophoresis as described earlier [109]. Briefly, 2 µl CSF in 10 µl sample buffer were loaded onto a 10 % Sodium-dodecylsulphate (SDS) Polyacrylamide gel containing type A gelatin from porcine skin (1 % v/v; Sigma, Buchs, Switzerland). Gels were run under non-reducing conditions at 100 V for 2.5 h. After incubation in SDS-removing buffer (1 % Triton X-

100, 3 changes for 1 h each), gelatin digestion was done for 18 h at 37°C in incubation buffer (10 mM $CaCl_2$, 50 mM Tris, 50 mM NaCl, pH 7.65). After staining the gel in Coomassie Blue, gelatinolytic activity was assessed by densitometric quantification of the gelatin lysis zones at 92 kDa (MMP-9) and at 72 kDa (MMP-2), using ImageJ software (NIH, http://rsb.info.nih.gov/ij/). Expression of MMP-9 is displayed as the ratio of MMP-9 and MMP-2. Statistical difference between the strains used was evaluated applying an unpaired *t* test.

4.3.6 Histopathology

Right fixed hemispheres were examined for neuronal injury in the cortex and for hippocampal apoptotic damage in the dentate gyrus. 45 µm thick cryosections were stained for Nissl substance with cresyl violet and used for all determinations. Quantification of apoptotic nuclei in the hippocampal dentate gyrus was performed as described earlier [149]. In brief, cells exhibiting characteristic histomorphological features of apoptosis were counted in 4 different slices spanning the hippocampus of the right hemisphere. Three visual fields in each of the two blades of the dentate gyrus were inspected for the appearance of cells showing morphological signs indicative of apoptosis (condensed, fragmented dark nuclei, apoptotic bodies). Each visual field was judged according to the following score: 0-5 cells = 0; 6-20 cells = 1; > 20 cells = 2. A mean value per animal was calculated from all inspected fields.

Cortical neuronal injury was expressed as the percentage of affected cortex, identified by decreased density of neurons or neuronal necrosis. The entire right hemisphere was evaluated by using a systematic uniform random sampling procedure, with a random start and a sampling frequency of 15 slices. Stained sections were scanned and total and damaged cortical surfaces were quantified by the software ImageJ, using a plug-in to lay a grid (http://rsb.info.nih.gov/ij/plugins/grid.html) over the scanned picture with a random offset and a surface of 1 mm^2 per cross. The volume of total cortex and the volume of the damaged tissue were calculated using the Cavalieri method [150]: multiplying the sum of all sectional areas (mm^2), determined by the total number of crosses in the regions of interest (whole cortex or region of decreased neuronal density) by the distance between successive sections (0.675 mm).

4.4The Mouse Models of Pneumococcal Disease

4.4.1 Model of Nasopharyngeal Colonization

Strains were grown to OD_{590nm}=0.6-0.7 and centrifuged to pellet bacterial cells. Depending of the experiment bacteria were resuspended in pyrogen-free saline (0.9% NaCl) to obtain bacterial concentration of 10^8-10^{10} CFU/ml. Groups of 8 week old CD1 female mice (5 per strain) were anaesthetized by i.p. injection of 75 µl of a xylazine and ketamine mixture [41]. Suspensions of bacteria (10µl) were inoculated through nostrils. Bacteria colonizing the nasopharynx were collected by expelling 50 µl saline solution through the trachea. Viable counts were determined on BAP supplemented with 5 µg/ml gentamycin.

4.4.2 Model of Intraperitoneal Sepsis

For the majority of the experiments eight week old female CD1 mice (Charles River Laboratories, Wilmington, MA, USA) were used. Time-course studies on the *in vivo* growth of bacteria were repeated three times. Mice were injected into the peritoneal cavity with 0.5 ml of the prepared inocula containing 10^6 CFU of bacteria. In each experiment blood from two mice per time point was collected by cardiac puncture and pooled. For bacterial titer determination serial dilutions of the blood samples were plated on 3 % sheep blood agar plates (BAP) supplemented with 5 µg/ml gentamicin and incubated at 37°C in a 5 % CO_2 atmosphere. Prior to the assay of serum cytokine levels, 10 µg/ml Mitomycin C was added to the blood samples followed by incubation for 1 h at 37°C, in order to kill the bacteria. Control experiments showed that Mitomycin C does not trigger cytokine production in uninfected blood samples. After centrifugation the serum was collected and stored at -80°C.

For experiments studying the effect of Toll-like receptor 2 (TLR-2) on the infection, 8 week old male B6.129-*Tlr2*^{tm1Kir}/J mice (The Jackson Laboratories, Bar Harbor, MN, USA) were used. For survival curves five B6.129-*Tlr2*^{tm1Kir}/J mice were compared to five C57BL/6 mice, which had the appropriate genetic background to serve as con-

trol. Both groups (n=5 animals) were infected with 10^5 CFU D39Cho$^-$ and survival of the mice was monitored.

4.4.3 Cytokine Determination in the Serum

The concentrations of cytokines from mouse sera were measured by Luminex (Millipore Corporation, St. Charles, MO, USA), according to the protocol of the manufacturer: 25 µl serum samples were incubated for 15 minutes with 25 µl of a serum diluent, followed by 2 h incubation with Beadmates coated with anti-cytokine mAbs. The plate was washed once and biotin-conjugated anti-cytokine mAbs were added for 1.5 h, followed by 30 min of incubation with Beadlyte streptavidin-PE. Samples were measured in duplicates by Luminex and analyzed using Beadview software (Millipore Corporation, St. Charles, MO, USA).

4.4.4 Maturation of Murine Splenic Dendritic Cells

To analyze the activation state of dendritic cells in mice infected with the different bacterial strains, one mouse spleen per bacterial strain was collected at timepoints 6 and 9 hours post infection. The tissues were transferred to Petri dishes containing Hanks medium supplemented with collagenase. After flushing with medium using a syringe, the spleen tissues were homogenized and incubated at 37°C for 25 minutes. Next, 100 µl of 0.5 M EDTA was added followed by incubation at 37°C for 5 min after which the cell suspensions were strained through a mesh and centrifuged. 1.5 ml ACK lysing Buffer (BioSource, Rockville, MD, USA) was added and incubated for 4 min at room temperature to allow erythrocyte lysis. To stop lysis 13 ml FACS buffer (PBS + 5 % Fetal calf serum) was added and cells were centrifuged for 10 min. CD11c$^+$CD8$^+$ and CD11c$^+$CD8$^-$ dendritic cells were analyzed by FACS for the presence of cell surface maturation markers CD80 and CD86. Two independent experiments were performed.

4.4.5 Induction of Protective Immunity

Mice were injected i.p. with 10^3 CFU of the avirulent D39Cho$^-$licA64 strain expressing the capsular polysaccharide type II (n=5 animals per group). Control mice (n=5 animals) were injected with saline. On day 10 after inoculation with the avirulent strain the animals were challenged with 10^4 CFU of the highly virulent strain D39Cho$^-$ and survival was monitored.

In a second experiment, animals were immunized i.p. with 10^4 CFU of the avirulent D39ChilicB31strain (n=5 animals per group). 5 control mice were injected with saline. On day 10 post infection the animals were challenged with a potentially lethal dose (10^4 and 10^6 CFU) of either of the highly virulent strains D39 (capsule type II) and SV36 (capsular type III) and survival was monitored. All mice that survived this first challenge were next challenged on day 25 with a lethal dose of 10^4 CFU of strain SV36.

4.5*In vitro* Assays

4.5.1 Maturation of human Monocyte-derived Dendritic Cells

Buffy coats purchased from the New York Blood Center were used as a source of mononuclear cells from healthy donors. Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood by density gradient centrifugation (Ficoll-Paque Plus; GE Healthcare). Monocytes were separated from the PBMC using CD14 microbeads (Miltenyi Biotech). Dendritic cells (DCs) were generated from purified blood monocytes as previously described [151]. The CD14$^+$ monocytes were cultured in RPMI1640 (Biowhittaker) supplemented with 1% plasma, in the presence of 800U/ml of GM-CSF (Immunex) and 1000 U/ml of IL-4 (R&D). The cultures were supplemented with cytokines on days 1, 3 and 5 of culture. On day 5, immature DCs were allowed to mature overnight with 100 ng/ml LPS (Sigma Aldrich).

Various bacteria as well as bacterial cell wall preparations were tested for their potential to induce DC maturation after incubation for 36 hours. Cultures of the choline containing strains D39Cho$^-$ and the isogenic choline-free derivative D39Cho$^-$licA64 were grown in C+Y medium at 37^{o}C. When the cell concentration in the cultures has

reached about 10^7 CFU per ml the cultures received 10 µg/ml Mitomycin C and were incubated for 1 h at 37°C to kill the bacteria, which was confirmed by plating on blood agar. 10^8 CFU equivalents of the non viable bacterial suspensions were applied to assay dendritic cell maturation.

Choline-free and choline containing cell walls were prepared and purified by the aforementioned published procedure from the non-encapsulated strain R6Cho$^-$ grown in Cden medium that was either free of choline or was supplemented with 5 µg/ml choline. Suspensions of cell walls were adjusted to the desired concentration in PBS and approximately 10^7 CFU equivalents were used in the assays.

Dendritic cell maturation was monitored by flow cytometry (FACS Calibur, BD) based on the presence of various maturation markers CD80, CD83 and CD86 (BD Biosciences). Each experiment was repeated three times using fresh batches of dendritic cells and freshly prepared mitomycin-killed bacteria.

4.5.2 Antimicrobial Activity of Murine Serum against *S. pneumoniae* ex vivo

Eight weeks old female CD 1 mice (n=7 per timepoint) (Charles River Laboratories, Wilmington, MA, USA) were infected i.p. with 2 x 10^4 CFU D39Cho$^-$licA64. Blood was collected at the desired time points by cardiac puncture of sacrificed animals. Serum was purified by centrifugation at 12000g for 3 min, pooled and stored at –80°C. To inactivate complement, serum was incubated at 56°C for 30min [131]. D39Cho$^-$ and D39Cho$^-$licA64 were grown in choline-free Cden medium (to assure chain growth) at 37ºC until OD_{590nm}= 0.7 and diluted in Cden. 10 µl of the prepared bacterial dilutions were inoculated into 90 µl purified serum to reach a bacterial titer of 10^4 CFU / ml serum and incubated at 37ºC. To block choline residues either IgAκ TEPC-15 [10 µg/ml] (Sigma, St. Louis, MO, USA) or human C-reactive protein CRP [30 µg/ml] (MP Biomedicals, Solon, OH, USA) were added to the serum. Viable counts of the bacteria at different time points were determined.

4.5.3 In vitro Killing of *S. pneumoniae* by the antimicrobial Peptide Nisin

The applied assay is based on a previously published protocol [41]. Bacteria were grown in C+Y medium at 37ºC until OD_{590nm}= 0.3 and Nisin (Sigma, St. Louis, MO, USA) was added to the culture at a concentration of 1 µg / ml. During further incubation at 37ºC the optical density was measured every 10 min for one hour. A Nisin-free culture served as control. The percentage of initial OD was calculated as the ratio of the values of Nisin-treated culture and the control culture for every time point. After 60 min serial dilutions of the cultures were plated and the ratio was determined. To block surface-bound choline residues of D39Cho$^-$ either mouse IgAκ TEPC-15 [10µg/ml] (Sigma, St. Louis, MO, USA) or human C-reactive protein CRP [30µg/ml] (MP Biomedicals, Solon, OH, USA) were added to a growing culture at OD_{590nm}=0.1 and incubated until OD_{590nm}=0.3 (corresponding to approximately 1.5 doubling times) prior to the addition of Nisin. A Nisin-free culture served as control and ratios were calculated as mentioned beforehand.

4.5.4 Pneumococcal Adherence to the Pharyngeal Cell line Detroit 562

The human pharyngeal cell line Detroit 562 was cultured in RPMI 1640 medium without phenol red and supplemented with 1 mM sodium pyruvate and 1% fetal bovine serum (FBS) in 24-well tissue culture plates. The plates were incubated at 37°C in 5% CO_2. *S. pneumoniae* strains were grown in C+Y medium to the logarithmic phase, diluted to the appropriate density in RPMI 1640 medium with 1% FBS and 1 ml aliquots were applied onto washed Detroit 562 monolayers. After incubation for 2 h at 37°C in 5% CO_2, the culture fluid was removed from each well and the monolayers were washed three times with PBS (pH 7.4). Pharyngeal cells were detached from the plates by treatment with 200 µl PBS (pH 7.4), 0.25% trypsin and 0.1% EDTA. Pharyngeal cells were next lysed by the addition of 800 µl of 0.025% Triton X-100, and appropriate dilutions were plated on BAP to count the numbers of bacteria adherent to and/or internalized by the pharyngeal cells.

For the invasion assay, monolayers were initially treated as for the adherence assay, but following the attachment of bacteria to the monolayers, 1 ml of RPMI 1640 medium with 1% FBS and penicillin (10 µg) and gentamicin (200 µg) was added to the

monolayers in order to kill bacterial cells attached to the surfaces of pharyngeal cells. The plates were incubated for 1 h at 37°C in 5% CO_2. After this step, the monolayers were washed three times with PBS (pH 7.4) to remove the antibiotics, pharyngeal cells were released and lysed, and lysates were plated on BAP to determine the numbers of internalized bacteria.

4.5.5 Detection of capsule-specific IgM antibodies with ELISA

Capsular-specific antibodies were quantified using a modified version of a previously described protocol [152]. 96-well plates (Nunc) were coated with 1µg/well of type II or type III capsular polysaccharide in carbonate buffer, pH 9.5 overnight at 4°C. Plates were washed with 0.05% Tween-20/PBS and blocked for 6 h with 1% BSA in PBS. Next, plates were washed and sera of mice, collected at day 10 after infection with D39ChiplicB31, were analyzed. Samples were plated out in duplicate serial three-fold dilutions, starting at a 1:6 dilution, and incubated over night at 4°C. The plates were washed, and goat anti-mouse IgM antibodies conjugated to HRP (Bethyl) were plated at 0.2µg/ml, and incubated for 4 h at room temperature. The plates were washed and developed with 3,3′,5,5′-tetramethylbenzidine (TMB) (KLP**,** Gaithersburg, MD) per manufacturer's instructions, and the absorbance read at 405nm.

4.5.6 Work with Nucleic Acids and Polymerase Chain Reaction (PCR)

All routine DNA works were performed by using standard methods [153]. PCR primer pheA (GCCTATTCATCAGCAGTTGATGGTGGTTCC) and primer hom (CTTGTCACCCTCTTTGCCATCTTGAAGGATTTGC) were synthesized at the custom primer facility of Invitrogen. Long Range PCR was perfomed using iProof High-Fidelity DNA polymerase (Bio-Rad Laboratories) according to the manucturers instructions. DNA sequencing was done at the Nano + Bio Center, University of Kaiserslautern. Nucleotide sequences were analyzed by using CloneManager software.

4.6 Materials

4.6.1 Instruments, Chemicals, Bacteria, Animals

Table 5: Laboratory Instruments

Function	Name/Model	Manufacturer
CO_2-Incubator	Forma Scientific Incubator	Thermo Fisher Scientific Inc., Waltham, MA, USA
Photometer	Spectronic 20D+	Thermo Fisher Scientific Inc., Waltham, MA, USA
Photometer	2800 UV/VIS	Unico, Dayton, NJ, USA
Centrifuge	CS-6R	Beckman Coulter, Fullerton, CA, USA
Centrifuge	Superspeed RC-2B	Thermo Fisher Scientific Inc., Waltham, MA, USA
Cell disintegrator	Savant FastPrep FP120	GMI, Ramsey, MI, USA
HPLC	LC-10AVP HPLC system	Shimadzu, Columbia, MD, USA
HPLC Column	Prontosil 120-3-C18-AQ column	Bischoff, Leonberg, GER
HPLC Column	Vydac 218TP54 column	W. R. Grace & Co.-Conn, Columbia, MD, USA
Mass Spectroscopy	4000 Q Trap mass spectrometer	Applied Biosystems, Foster City, CA, USA
Cytokine Determination	Luminex	Millipore Corporation, St. Charles, MO, USA
Cytokine Determination	Lincoplex, Microsphere-based multiplex assays	Linco Research Inc., St Charles, MA, USA
Fluorescence-activated cell sorting	FACS Calibur	BD Biosciences, Franklin Lakes, NJ, USA

Table 6: Enzymes, Antibiotics, Isotopes, Immune Molecules

Name	Company/Supplier	Location
Amidase	kindly provided by RU[1]	New York, USA
Cellosyl	kindly provided by Hoechst	Frankfurt, GER
Collagenase	kindly provided by RU[1]	New York, USA
Myeloperoxidase	Sigma	St. Louis, MO, USA
iProof High-Fidelity DNA Polymerase	Bio-Rad Laboratories	Hercules, CA, USA
Gentamicin	Sigma	St. Louis, MO, USA
Mitomycin C	Sigma	St. Louis, MO, USA
Nisin	Sigma	St. Louis, MO, USA
Penicillin	Sigma	St. Louis, MO, USA
^{3}H-Choline	Amersham Biosciences	Pittsburgh, PA, USA
C-reactive Protein, human	MP Biomedicals	Solon, OH, USA
IgAκ TEPC-15 antibody	Sigma	St. Louis, MO, USA

[1] RU: The Rockefeller University

Table 7: Bacterial strains

Strain name	Description	Choline auxotrophy	Reference/ source
Pen6	Pen^{R} transformant of R6Hex with donor DNA from penicillin-resistant strain 8249.	+	[154]
Pen6*adr*	Mariner insertion mutant in *adr* in Pen6	+	[104]
R36ASIII	Laboratory strain expressing type 3 capsule	+	RU[1]
R36ASIII*adr*	R36ASIII insertion mutant in gene *adr*	+	RU[1]
R6	Laboratory strain	+	RU[1]
R6Cho^{-}	Choline-independent derivative of R6	-	[38]
R6Cho^{-} Δ*licD1D2*	*licD1-licD2* deletion mutant of R6Cho^{-}	-	[27]
R6Cho^{-}Δ*lic2*	*lic2* operon deletion mutant of R6Cho^{-}	-	[27]
D39	Type 2 capsular virulent *S. pneumoniae strain*	+	RU[1]
D39Cho^{-}	Choline-independent derivative of D39	-	[41]
D39Cho^{-}licA64	D39Cho^{-} derivative with insertion duplication deletion in gene *licA*	-	[41]
D39Cho^{-} Δ*licD1D2*	*licD1-licD2* insertion duplication deletion mutant of R6Cho^{-}	-	[27]

D39Cho$^-$Δ*lic2*	*lic2* operon insertion duplication deletion mutant of R6Cho$^-$	-	[27]
D39Chi	Choline-independent derivative of D39 carrying G700T point mutation in the *tacF* gene.	-	[28]
D39ChilicA65	D39Chi derivative with insertion duplication deletion in gene *licA*	-	[28]
D39ChilicB31	D39Chi derivate with insertion duplication deletion in gene *licB*	-	[28]
SV36	Clinical isolate with type III capsule	+	[16]

[1] RU: The Rockefeller University collection

Table 8: Animals / Cell lines

Name	Company/Supplier	Location
Wistar rats	Charles River Laboratories	Sulzfeld, GER
CD1 mice	Charles River Laboratories	Wilmington, MA, USA
C57BL/6 mice	The Jackson Laboratories	Bar Harbor, MN, USA
B6.129-*Tlr2*^{tm1Kir}/J mice	The Jackson Laboratories	Bar Harbor, MN, USA
Detroit 562 pharyngeal cells	ATCC	Manassas, VA, USA
Buffy coats	New York Blood Center	New York, USA

4.6.2 Culture media

Table 9: Non-commercial Bacterial Growth Media

C+Y Medium	
C Medium	453 ml
Difco yeast extract (5%)	10 ml
Total	463 ml

C Medium	
PreC	400 ml
Supplement	13 ml
Glutamine (1 mg/ ml)	10 ml
Adams III	10 ml
Pyruvat (2%)	5 ml
KPO_4-Puffer 1M, pH 8 or 6,6 (pH 7,0 preserves O-Acetyl-group)	15 ml
Total	453 ml

PreC	
Sodium-Acetat	14,5 g
Difco Casamino Acids	60 g
L-Tryptophan	0,06 g
L-Cystein	0,6 g
Total (add MilliQ)	12000 ml

3 in 1 Salts	
$MgCl_2$ ($6H_2O$)	100 g
$CaCl_2$	0,5 g
$MnSO_4$ ($4H_2O$) (0,1 M)	0,2 ml
Total (add MilliQ)	1000 ml

Supplement	
3 in 1 Salts	60 ml
Sucrose (50%)	6 ml
Glucose (20%)	120 ml
Adenosine (2 mg/ml)	120 ml
Uridine (2 mg/ml)	120 ml
Total	426 ml

Adams I	
Nicotinic Acid	30 mg
Pyridoxine	35 mg
Ca-Pantothenate	120 mg
Thiamine-Hcl	32 mg
Riboflavin	14 mg
Biotin (0,5 mg/ml)	0,06 ml
Total (add MilliQ)	200 ml

Adams II	
$FeSO_4(7H_2O)$	50 mg
$CuSO_4(5H_2O)$	50 mg
$ZnSO_4(7H_2O)$	50 mg
$MnSO_4(4H_2O)$	20 mg
Hcl	1 ml
Total (add MilliQ)	100 ml

Adams III	
Asparagine	800 mg
Choline	80 mg
$CaCl_2$ (1 %)	0,64 ml
Adams I	64 ml
Adams II	16 ml
Total (add MilliQ)	400 ml

Cden Medium	
Cden Base	200 ml
HTA	50 ml
Glutamine (1 mg/ml)	10 ml
Vitamins	10 ml
Pyruvate (2%)	5 ml
SAC	40 ml
Supplement	13 ml
KPO_4-Buffer (1 M)	15 ml
Leucin (10 mg/ml)	10 ml
Phenylalanine (10 mg/ml)	5 ml
Lysine (10 mg/ml)	9 ml
Choline (1 mg/ml)	2 ml
Total (add MilliQ)	400 ml

HTA	
Histidne	640 mg
Tyrosine	122 mg
Arginine	800 mg
Total (add MilliQ)	1000 ml

Cden Base	
L-Glycine	190 mg
L-Alanine	350 mg
L-Valine	720 mg
L-Isoleucin	760 mg
L-Proline	1160 mg
L-Serine	590 mg
L-Threonine	450 mg
L-Methionine	310 mg
L-Tryptophan	140 mg
L-Aspartic acid	720 mg
L-Glutamic acid	2200 mg

L-Cysteine	150 mg
Adjust to pH 7.0 with 10M NaOH	
Total (add MilliQ)	2000 ml

Vitamins	
Adams I	12.8 ml
Asparagine (5 mg/ml)	32 ml
Total (add MilliQ)	80.8 ml

SAC	
Na-Acetate ($3H_2O$) (0.2%)	2 g
NaCl	2 g
Total (add MilliQ)	100 ml

Phosphate Buffer KPO_4 pH8.0	
KH_2PO_4 (1M)	26.5 ml
K_2HPO_4 (1M)	473 ml
Total (add MilliQ)	500 ml

References

[1] Avery, O. T.; MacLeod, C. M. and McCarty, M. (1979): Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Inductions of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III, J Exp Med (vol. 149), No. 2, pp. 297-326. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=33226

[2] Tomasz, A. and Hotchkiss, R. D. (1964): Regulation Of The Transformability Of Pneumococcal Cultures By Macromolecular Cell Products, Proc Natl Acad Sci U S A (vol. 51), pp. 480-7. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14171462

[3] WHO (2007): Pneumococcal conjugate vaccine for childhood immunization--WHO position paper, Wkly Epidemiol Rec (vol. 82), No. 12, pp. 93-104. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17380597

[4] Brundage, J. F. (2006): Interactions between influenza and bacterial respiratory pathogens: implications for pandemic preparedness, Lancet Infect Dis (vol. 6), No. 5, pp. 303-12. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16631551

[5] Klugman, K. P.; Madhi, S. A. and Feldman, C. (2007): HIV and pneumococcal disease, Curr Opin Infect Dis (vol. 20), No. 1, pp. 11-5. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17197876

[6] Mäkelä, H. and Butler, J.B. (2008): History of Pneumococcal Immunization, Siber, G.R., Pneumococcal vaccines pp. 19-29, ASM Press, Washington.

[7] Francis, T. and Tillett, W. (1930): Cutaneous reactions in pneumonia. The development of antibodies following the intradermal injection of type-specific polysaccharides, J Exp. Med. (vol. 52), pp. 573-585.

[8] Jacobs, M. R.; Koornhof, H. J.; Robins-Browne, R. M.; Stevenson, C. M.; Vermaak, Z. A.; Freiman, I.; Miller, G. B.; Witcomb, M. A.; Isaacson, M.; Ward, J. I. and Austrian, R. (1978): Emergence of multiply resistant pneumococci, N Engl J Med (vol. 299), No. 14, pp. 735-40. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=29219

[9] Austrian, R. (2000): The Enduring Pneumococcus: Unfinished Business and Opportunities for the Future, Tomasz, Alexander, *In* Streptococcus pneumoniae. Molecular Biology & Mechanism of Disease, Mary Ann Liebert, Inc., New York.

[10] Frazao, N.; Brito-Avo, A.; Simas, C.; Saldanha, J.; Mato, R.; Nunes, S.; Sousa, N. G.; Carrico, J. A.; Almeida, J. S.; Santos-Sanches, I. and de Lencastre, H. (2005): Effect of the seven-valent conjugate pneumococcal vaccine on carriage and drug resistance of *Streptococcus pneumoniae* in healthy children attending day-care centers in Lisbon, Pediatr Infect Dis J (vol. 24), No. 3, pp. 243-52. URL:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15750461

[11] Filipe, S. R.; Severina, E. and Tomasz, A. (2001): The role of murMN operon in penicillin resistance and antibiotic tolerance of *Streptococcus pneumoniae*, Microb Drug Resist (vol. 7), No. 4, pp. 303-16. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11822770

[12] Vollmer, W. and Tomasz, A. (2000): The pgdA gene encodes for a peptidoglycan N-acetylglucosamine deacetylase in *Streptococcus pneumoniae*, J Biol Chem (vol. 275), No. 27, pp. 20496-501. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10781617

[13] Park, I. H.; Pritchard, D. G.; Cartee, R.; Brandao, A.; Brandileone, M. C. and Nahm, M. H. (2007): Discovery of a new capsular serotype (6C) within serogroup 6 of *Streptococcus pneumoniae*, J Clin Microbiol (vol. 45), No. 4, pp. 1225-33. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17267625

[14] Kamerling, J.P. (2000): Pneumococcal Polysaccharides: A Chemical View, Tomasz, A., Streptococcus pneumoniae - Molecular Biology and Mechanism of Disease pp. 81-114, Mary Ann Liebert, Inc. publishers, New York.

[15] Yother, J.; Bentley, S.D. and Hennessey JR., J.P. (2008): Genetics, Biosynthesis, and Chemistry of Pneumococcal Capsular Polysaccharides, Siber, G.R., Pneumococcal Vaccines: The Impact of Conjugate Vaccine pp. 33-46, ASM Press, Washington D.C.

[16] Nesin, M.; Ramirez, M. and Tomasz, A. (1998): Capsular transformation of a multidrug-resistant *Streptococcus pneumoniae* in vivo, J Infect Dis (vol. 177), No. 3, pp. 707-13. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9498451

[17] Kovacs, M.; Halfmann, A.; Fedtke, I.; Heintz, M.; Peschel, A.; Vollmer, W.; Hakenbeck, R. and Bruckner, R. (2006): A functional dlt operon, encoding proteins required for incorporation of d-alanine in teichoic acids in gram-positive bacteria, confers resistance to cationic antimicrobial peptides in *Streptococcus pneumoniae*, J Bacteriol (vol. 188), No. 16, pp. 5797-805. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16885447

[18] Tomasz, A. (1967): Choline in the cell wall of a bacterium: novel type of polymer-linked choline in Pneumococcus, Science (vol. 157), No. 789, pp. 694-7. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=4381896

[19] Fischer, W. (2000): Phosphocholine of pneumococcal teichoic acids: role in bacterial physiology and pneumococcal infection, Res Microbiol (vol. 151), No. 6, pp. 421-7. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10961454

[20] Rane, L. and Subbarow, Y. (1940): Nutritional Requirements of the Pneumococcus: I. Growth Factors for Types I, II, V, VII, VIII, J Bacteriol (vol. 40), No. 5, pp. 695-704. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16560380

[21] Baur, S.; Marles-Wright, J.; Buckenmaier, S.; Lewis, R. J. and Vollmer, W. (2009): Synthesis of CDP-activated ribitol for teichoic acid precursors in *Streptococcus pneumoniae,* J Bacteriol (vol. 191), No. 4, pp. 1200-10. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19074383

[22] Bean, B. and Tomasz, A. (1977): Choline metabolism in pneumococci, J Bacteriol (vol. 130), No. 1, pp. 571-4. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15988

[23] Whiting, G. C. and Gillespie, S. H. (1996): Incorporation of choline into *Streptococcus pneumoniae* cell wall antigens: evidence for choline kinase activity, FEMS Microbiol Lett (vol. 138), No. 2-3, pp. 141-5. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9026440

[24] Whiting, G. C. and Gillespie, S. H. (1996): Investigation of a choline phosphate synthesis pathway in *Streptococcus pneumoniae*: evidence for choline phosphate cytidylyltransferase activity, FEMS Microbiol Lett (vol. 143), No. 2-3, pp. 279-84. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8837483

[25] Rock, C. O.; Heath, R. J.; Park, H. W. and Jackowski, S. (2001): The licC gene of *Streptococcus pneumoniae* encodes a CTP:phosphocholine cytidylyltransferase, J Bacteriol (vol. 183), No. 16, pp. 4927-31. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11466299

[26] Zhang, J. R.; Idanpaan-Heikkila, I.; Fischer, W. and Tuomanen, E. I. (1999): Pneumococcal licD2 gene is involved in phosphorylcholine metabolism, Mol Microbiol (vol. 31), No. 5, pp. 1477-88. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10200966

[27] Kharat, A. S.; Denapaite, D.; Gehre, F.; Bruckner, R.; Vollmer, W.; Hakenbeck, R. and Tomasz, A. (2008): Different pathways of choline metabolism in two choline-independent strains of *Streptococcus pneumoniae* and their impact on virulence, J Bacteriol (vol. 190), No. 17, pp. 5907-14. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18621904

[28] Damjanovic, M.; Kharat, A. S.; Eberhardt, A.; Tomasz, A. and Vollmer, W. (2007): The essential tacF gene is responsible for the choline-dependent growth phenotype of *Streptococcus pneumoniae*, J Bacteriol (vol. 189), No. 19, pp. 7105-11. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17660291

[29] Vollmer, W. and Tomasz, A. (2001): Identification of the teichoic acid phosphorylcholine esterase in *Streptococcus pneumoniae*, Mol Microbiol (vol. 39), No. 6, pp. 1610-22. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11260477

[30] Ware, D.; Watt, J. and Swiatlo, E. (2005): Utilization of putrescine by *Streptococcus pneumoniae* during growth in choline-limited medium, J Microbiol (vol. 43), No. 5, pp. 398-405. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16273030

[31] Tomasz, A. (1968): Biological consequences of the replacement of choline by ethanolamine in the cell wall of Pneumococcus: chain formation, loss of transformability, and loss of autolysis, Proc Natl Acad Sci U S A (vol. 59), No. 1, pp. 86-93. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=4385544

[32] Garcia, J. L.; Sanchez-Beato, A. R.; Medrano, F. J. and Lopez, R. (1998): Versatility of choline-binding domain, Microb Drug Resist (vol. 4), No. 1, pp. 25-36. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9533722

[33] Swiatlo, E.; McDaniel, L. S. and Briles, D. E. (2004): Choline-Binding Proteins, Tuomanen, E. I., The Pneumococcus pp. 49-60, ASM Press, Washington D.C.

[34] Garcia, P.; Gonzalez, M. P.; Garcia, E.; Lopez, R. and Garcia, J. L. (1999): LytB, a novel pneumococcal murein hydrolase essential for cell separation, Mol Microbiol (vol. 31), No. 4, pp. 1275-81. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10096093

[35] Garcia, P.; Paz Gonzalez, M.; Garcia, E.; Garcia, J. L. and Lopez, R. (1999): The molecular characterization of the first autolytic lysozyme of *Streptococcus pneumoniae* reveals evolutionary mobile domains, Mol Microbiol (vol. 33), No. 1, pp. 128-38. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10411730

[36] Molina, R.; Gonzalez, A.; Stelter, M.; Perez-Dorado, I.; Kahn, R.; Morales, M.; Campuzano, S.; Campillo, N. E.; Mobashery, S.; Garcia, J. L.; Garcia, P. and Hermoso, J. A. (2009): Crystal structure of CbpF, a bifunctional choline-binding protein and autolysis regulator from *Streptococcus pneumoniae*, EMBO Rep. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19165143

[37] Kausmally, L.; Johnsborg, O.; Lunde, M.; Knutsen, E. and Havarstein, L. S. (2005): Choline-binding protein D (CbpD) in *Streptococcus pneumoniae* is essential for competence-induced cell lysis, J Bacteriol (vol. 187), No. 13, pp. 4338-45. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15968042

[38] Severin, A.; Horne, D. and Tomasz, A. (1997): Autolysis and cell wall degradation in a choline-independent strain of *Streptococcus pneumoniae*, Microb Drug Resist (vol. 3), No. 4, pp. 391-400. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9442493

[39] Yother, J.; Leopold, K.; White, J. and Fischer, W. (1998): Generation and properties of a *Streptococcus pneumoniae* mutant which does not require choline or analogs for growth, J Bacteriol (vol. 180), No. 8, pp. 2093-101. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9555891

[40] Gonzalez, A.; Llull, D.; Morales, M.; Garcia, P. and Garcia, E. (2008): Mutations in the tacF gene of clinical strains and laboratory transformants of *Streptococcus pneumoniae*: impact on choline auxotrophy and growth rate, J Bacteriol (vol. 190), No. 12, pp. 4129-38. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18424523

[41] Kharat, A. S. and Tomasz, A. (2006): Drastic reduction in the virulence of *Streptococcus pneumoniae* expressing type 2 capsular polysaccharide but lacking choline residues in the cell wall, Mol Microbiol (vol. 60), No. 1, pp. 93-107. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16556223

[42] Kharat, A. S. and Tomasz, A. (2003): Inactivation of the srtA gene affects localization of surface proteins and decreases adhesion of *Streptococcus pneumoniae* to human pharyngeal cells in vitro, Infect Immun (vol. 71), No. 5, pp. 2758-65. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12704150

[43] Bergmann, S. and Hammerschmidt, S. (2006): Versatility of pneumococcal surface proteins, Microbiology (vol. 152), No. Pt 2, pp. 295-303. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16436417

[44] Giebink, S.G. (2000): Otitis Media: The Chinchilla Model, Tomasz, A., *Streptococcus Pneumoniae*. Molecular Biology & Mechanisms of Disease pp. 343-358, Mary Ann Liebert, Larchmont.

[45] Janeway, C.A.; Travers, P.; Walport, M. and Shlomchik, M.J. (2005): Immunobiology, 6th. ed., Garland Science Publishing, New York.

[46] Lyczak, J. B. (2004): Innate Immunity, Pier, G.B., Lyczak, J.B., Wetzler, L.M., Immunology, Infection, and Immunity pp. 29-46, ASM Press, Washington, D.C.

[47] Bergman, P.; Johansson, L.; Wan, H.; Jones, A.; Gallo, R. L.; Gudmundsson, G. H.; Hokfelt, T.; Jonsson, A. B. and Agerberth, B. (2006): Induction of the antimicrobial peptide CRAMP in the blood-brain barrier and meninges after meningococcal infection, Infect Immun (vol. 74), No. 12, pp. 6982-91. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17030578

[48] Ihi, T.; Nakazato, M.; Mukae, H. and Matsukura, S. (1997): Elevated concentrations of human neutrophil peptides in plasma, blood, and body fluids from

patients with infections, Clin Infect Dis (vol. 25), No. 5, pp. 1134-40. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9402371

[49] Maffei, F. A.; Heine, R. P.; Whalen, M. J.; Mortimer, L. F. and Carcillo, J. A. (1999): Levels of antimicrobial molecules defensin and lactoferrin are elevated in the cerebrospinal fluid of children with meningitis, Pediatrics (vol. 103), No. 5 Pt 1, pp. 987-92. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10224177

[50] Moser, C.; Weiner, D. J.; Lysenko, E.; Bals, R.; Weiser, J. N. and Wilson, J. M. (2002): beta-Defensin 1 contributes to pulmonary innate immunity in mice, Infect Immun (vol. 70), No. 6, pp. 3068-72. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12010999

[51] Brandenburg, L. O.; Varoga, D.; Nicolaeva, N.; Leib, S. L.; Wilms, H.; Podschun, R.; Wruck, C. J.; Schroder, J. M.; Pufe, T. and Lucius, R. (2008): Role of glial cells in the functional expression of LL-37/rat cathelin-related antimicrobial peptide in meningitis, J Neuropathol Exp Neurol (vol. 67), No. 11, pp. 1041-54. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18957897

[52] Peschel, A. (2002): How do bacteria resist human antimicrobial peptides? Trends Microbiol (vol. 10), No. 4, pp. 179-86. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11912025

[53] Woof, J. M. and Kerr, M. A. (2006): The function of immunoglobulin A in immunity, J Pathol (vol. 208), No. 2, pp. 270-82. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16362985

[54] Briles, D. E.; Nahm, M.; Schroer, K.; Davie, J.; Baker, P.; Kearney, J. and Barletta, R. (1981): Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 *Streptococcus pneumoniae*, J Exp Med (vol. 153), No. 3, pp. 694-705. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7252411

[55] Hostetter, M.K. (2004): Interactions of *Streptococcus pneumoniae* with the proteins of the complement pathways, Tuomanen, E. I., The Pneumococcus pp. 201-210, ASM Press, Washington, D.C.

[56] Janeway, C. A., Jr. and Medzhitov, R. (2002): Innate immune recognition, Annu Rev Immunol (vol. 20), pp. 197-216. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11861602

[57] Joiner, K.; Brown, E.; Hammer, C.; Warren, K. and Frank, M. (1983): Studies on the mechanism of bacterial resistance to complement-mediated killing. III. C5b-9 deposits stably on rough and type 7 *S. pneumoniae* without causing bacterial killing, J Immunol (vol. 130), No. 2, pp. 845-9. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=6848598

[58] Mold, C. and Du Clos, T. W. (2006): C-reactive protein increases cytokine responses to *Streptococcus pneumoniae* through interactions with Fgamma receptors, J Immunol (vol. 176), No. 12, pp. 7598-604. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16751406

[59] Gould, J. M. and Weiser, J. N. (2001): Expression of C-reactive protein in the human respiratory tract, Infect Immun (vol. 69), No. 3, pp. 1747-54. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11179352

[60] Volanakis, J. E. and Kaplan, M. H. (1971): Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide, Proc Soc Exp Biol Med (vol. 136), No. 2, pp. 612-4. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=4395924

[61] Christner, R. B. and Mortensen, R. F. (1994): Binding of human serum amyloid P-component to phosphocholine, Arch Biochem Biophys (vol. 314), No. 2, pp. 337-43. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7979374

[62] Yuste, J.; Botto, M.; Bottoms, S. E. and Brown, J. S. (2007): Serum amyloid P aids complement-mediated immunity to *Streptococcus pneumoniae*, PLoS Pathog (vol. 3), No. 9, pp. 1208-19. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17845072

[63] Kaplan, M. H. and Volanakis, J. E. (1974): Interaction of C-reactive protein complexes with the complement system. I. Consumption of human complement associated with the reaction of C-reactive protein with pneumococcal C-polysaccharide and with the choline phosphatides, lecithin and sphingomyelin, J Immunol (vol. 112), No. 6, pp. 2135-47. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=4151108

[64] Mortensen, R. F. and Duszkiewicz, J. A. (1977): Mediation of CRP-dependent phagocytosis through mouse macrophage Fc-receptors, J Immunol (vol. 119), No. 5, pp. 1611-6. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=915271

[65] Mold, C.; Rodic-Polic, B. and Du Clos, T. W. (2002): Protection from *Streptococcus pneumoniae* infection by C-reactive protein and natural antibody requires complement but not Fc gamma receptors, J Immunol (vol. 168), No. 12, pp. 6375-81. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12055255

[66] Zamze, S.; Martinez-Pomares, L.; Jones, H.; Taylor, P. R.; Stillion, R. J.; Gordon, S. and Wong, S. Y. (2002): Recognition of bacterial capsular polysaccharides and lipopolysaccharides by the macrophage mannose receptor, J Biol Chem (vol. 277), No. 44, pp. 41613-23. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12196537

[67] Greenberg, J. W.; Fischer, W. and Joiner, K. A. (1996): Influence of lipoteichoic acid structure on recognition by the macrophage scavenger receptor, Infect Immun (vol. 64), No. 8, pp. 3318-25. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8757870

[68] Arredouani, M.; Yang, Z.; Ning, Y.; Qin, G.; Soininen, R.; Tryggvason, K. and Kobzik, L. (2004): The scavenger receptor MARCO is required for lung defense against pneumococcal pneumonia and inhaled particles, J Exp Med (vol. 200), No. 2, pp. 267-72. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15263032

[69] Kang, Y. S.; Kim, J. Y.; Bruening, S. A.; Pack, M.; Charalambous, A.; Pritsker, A.; Moran, T. M.; Loeffler, J. M.; Steinman, R. M. and Park, C. G. (2004): The C-type lectin SIGN-R1 mediates uptake of the capsular polysaccharide of *Streptococcus pneumoniae* in the marginal zone of mouse spleen, Proc Natl Acad Sci U S A (vol. 101), No. 1, pp. 215-20. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14694198

[70] Brinkmann, V.; Reichard, U.; Goosmann, C.; Fauler, B.; Uhlemann, Y.; Weiss, D. S.; Weinrauch, Y. and Zychlinsky, A. (2004): Neutrophil extracellular traps kill bacteria, Science (vol. 303), No. 5663, pp. 1532-5. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15001782

[71] Brinkmann, V. and Zychlinsky, A. (2007): Beneficial suicide: why neutrophils die to make NETs, Nat Rev Microbiol (vol. 5), No. 8, pp. 577-82. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17632569

[72] Malley, R.; Henneke, P.; Morse, S. C.; Cieslewicz, M. J.; Lipsitch, M.; Thompson, C. M.; Kurt-Jones, E.; Paton, J. C.; Wessels, M. R. and Golenbock, D. T. (2003): Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection, Proc Natl Acad Sci U S A (vol. 100), No. 4, pp. 1966-71. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12569171

[73] Hemmi, H.; Takeuchi, O.; Kawai, T.; Kaisho, T.; Sato, S.; Sanjo, H.; Matsumoto, M.; Hoshino, K.; Wagner, H.; Takeda, K. and Akira, S. (2000): A Toll-like receptor recognizes bacterial DNA, Nature (vol. 408), No. 6813, pp. 740-5. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11130078

[74] Girardin, S. E.; Boneca, I. G.; Viala, J.; Chamaillard, M.; Labigne, A.; Thomas, G.; Philpott, D. J. and Sansonetti, P. J. (2003): Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection, J Biol Chem (vol. 278), No. 11, pp. 8869-72. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12527755

[75] Wang, Z. M.; Li, X.; Cocklin, R. R.; Wang, M.; Wang, M.; Fukase, K.; Inamura, S.; Kusumoto, S.; Gupta, D. and Dziarski, R. (2003): Human peptidoglycan

recognition protein-L is an N-acetylmuramoyl-L-alanine amidase, J Biol Chem (vol. 278), No. 49, pp. 49044-52. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14506276

[76] Majcherczyk, P. A. and Moreillon, P. (2004): Inflammation and Host Defense, Tuomanen, E., The Pneumococcus pp. 183-200, ASM Press, Washington D.C.

[77] Balachandran, P.; Brooks-Walter, A.; Virolainen-Julkunen, A.; Hollingshead, S. K. and Briles, D. E. (2002): Role of pneumococcal surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of *Streptococcus pneumoniae*, Infect Immun (vol. 70), No. 5, pp. 2526-34. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11953392

[78] Briles, D. E.; Ades, E.; Paton, J. C.; Sampson, J. S.; Carlone, G. M.; Huebner, R. C.; Virolainen, A.; Swiatlo, E. and Hollingshead, S. K. (2000): Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*, Infect Immun (vol. 68), No. 2, pp. 796-800. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10639448

[79] Johnson, S. E.; Dykes, J. K.; Jue, D. L.; Sampson, J. S.; Carlone, G. M. and Ades, E. W. (2002): Inhibition of pneumococcal carriage in mice by subcutaneous immunization with peptides from the common surface protein pneumococcal surface adhesin a, J Infect Dis (vol. 185), No. 4, pp. 489-96. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11865401

[80] Snapper C.M.; Colino C.; Khan A.Q. and Z.Q., Wu (2004): The humoral immune response to *Streptococcus pneumoniae*, Tuomanen, E., The Pneumococcus pp. 367-381, ASM Press, Washingtopn D.C.

[81] Weiser, J. N. (1998): Phase variation in colony opacity by *Streptococcus pneumoniae*, Microb Drug Resist (vol. 4), No. 2, pp. 129-35. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9651000

[82] Oggioni, M. R.; Trappetti, C.; Kadioglu, A.; Cassone, M.; Iannelli, F.; Ricci, S.; Andrew, P. W. and Pozzi, G. (2006): Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis, Mol Microbiol (vol. 61), No. 5, pp. 1196-210. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16925554

[83] Nelson, A. L.; Roche, A. M.; Gould, J. M.; Chim, K.; Ratner, A. J. and Weiser, J. N. (2007): Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance, Infect Immun (vol. 75), No. 1, pp. 83-90. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17088346

[84] Selva, L.; Viana, D.; Regev-Yochay, G.; Trzcinski, K.; Corpa, J. M.; Lasa, I.; Novick, R. P. and Penades, J. R. (2009): Killing niche competitors by remote-control bacteriophage induction, Proc Natl Acad Sci U S A (vol. 106), No. 4,

pp. 1234-8. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19141630

[85] Cundell, D. R.; Gerard, N. P.; Gerard, C.; Idanpaan-Heikkila, I. and Tuomanen, E. I. (1995): *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor, Nature (vol. 377), No. 6548, pp. 435-8. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7566121

[86] Andersson, B.; Dahmen, J.; Frejd, T.; Leffler, H.; Magnusson, G.; Noori, G. and Eden, C. S. (1983): Identification of an active disaccharide unit of a glycoconjugate receptor for pneumococci attaching to human pharyngeal epithelial cells, J Exp Med (vol. 158), No. 2, pp. 559-70. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=6886624

[87] Cundell, D. R. and Tuomanen, E. I. (1994): Receptor specificity of adherence of *Streptococcus pneumoniae* to human type-II pneumocytes and vascular endothelial cells in vitro, Microb Pathog (vol. 17), No. 6, pp. 361-74. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7752878

[88] Barthelson, R.; Mobasseri, A.; Zopf, D. and Simon, P. (1998): Adherence of *Streptococcus pneumoniae* to respiratory epithelial cells is inhibited by sialylated oligosaccharides, Infect Immun (vol. 66), No. 4, pp. 1439-44. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9529065

[89] Krivan, H. C.; Roberts, D. D. and Ginsburg, V. (1988): Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAc beta 1-4Gal found in some glycolipids, Proc Natl Acad Sci U S A (vol. 85), No. 16, pp. 6157-61. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=3413084

[90] Barocchi, M. A.; Ries, J.; Zogaj, X.; Hemsley, C.; Albiger, B.; Kanth, A.; Dahlberg, S.; Fernebro, J.; Moschioni, M.; Masignani, V.; Hultenby, K.; Taddei, A. R.; Beiter, K.; Wartha, F.; von Euler, A.; Covacci, A.; Holden, D. W.; Normark, S.; Rappuoli, R. and Henriques-Normark, B. (2006): A pneumococcal pilus influences virulence and host inflammatory responses, Proc Natl Acad Sci U S A. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16481624

[91] Basset, A.; Trzcinski, K.; Hermos, C.; O'Brien, K. L.; Reid, R.; Santosham, M.; McAdam, A. J.; Lipsitch, M. and Malley, R. (2007): Association of the pneumococcal pilus with certain capsular serotypes but not with increased virulence, J Clin Microbiol (vol. 45), No. 6, pp. 1684-9. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17392439

[92] Munoz-Elias, E. J.; Marcano, J. and Camilli, A. (2008): Isolation of *Streptococcus pneumoniae* biofilm mutants and their characterization during nasopharyngeal colonization, Infect Immun (vol. 76), No. 11, pp. 5049-61. URL:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18794289

[93] Zysk, G.; Bejo, L.; Schneider-Wald, B. K.; Nau, R. and Heinz, H. (2000): Induction of necrosis and apoptosis of neutrophil granulocytes by *Streptococcus pneumoniae*, Clin Exp Immunol (vol. 122), No. 1, pp. 61-6. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11012619

[94] Dockrell, D. H.; Lee, M.; Lynch, D. H. and Read, R. C. (2001): Immune-mediated phagocytosis and killing of *Streptococcus pneumoniae* are associated with direct and bystander macrophage apoptosis, J Infect Dis (vol. 184), No. 6, pp. 713-22. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11517432

[95] Rubins, J. B.; Duane, P. G.; Charboneau, D. and Janoff, E. N. (1992): Toxicity of pneumolysin to pulmonary endothelial cells in vitro, Infect Immun (vol. 60), No. 5, pp. 1740-6. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=1563759

[96] Rubins, J. B.; Duane, P. G.; Clawson, D.; Charboneau, D.; Young, J. and Niewoehner, D. E. (1993): Toxicity of pneumolysin to pulmonary alveolar epithelial cells, Infect Immun (vol. 61), No. 4, pp. 1352-8. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8454338

[97] Zysk, G.; Schneider-Wald, B. K.; Hwang, J. H.; Bejo, L.; Kim, K. S.; Mitchell, T. J.; Hakenbeck, R. and Heinz, H. P. (2001): Pneumolysin is the main inducer of cytotoxicity to brain microvascular endothelial cells caused by *Streptococcus pneumoniae*, Infect Immun (vol. 69), No. 2, pp. 845-52. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11159977

[98] Braun, J. S.; Sublett, J. E.; Freyer, D.; Mitchell, T. J.; Cleveland, J. L.; Tuomanen, E. I. and Weber, J. R. (2002): Pneumococcal pneumolysin and H(2)O(2) mediate brain cell apoptosis during meningitis, J Clin Invest (vol. 109), No. 1, pp. 19-27. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11781347

[99] Balachandran, P.; Hollingshead, S. K.; Paton, J. C. and Briles, D. E. (2001): The autolytic enzyme LytA of *Streptococcus pneumoniae* is not responsible for releasing pneumolysin, J Bacteriol (vol. 183), No. 10, pp. 3108-16. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11325939

[100] Canvin, J. R.; Marvin, A. P.; Sivakumaran, M.; Paton, J. C.; Boulnois, G. J.; Andrew, P. W. and Mitchell, T. J. (1995): The role of pneumolysin and autolysin in the pathology of pneumonia and septicemia in mice infected with a type 2 pneumococcus, J Infect Dis (vol. 172), No. 1, pp. 119-23. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7797901

[101] Tomasz, A.; Moreillon, P. and Pozzi, G. (1988): Insertional inactivation of the major autolysin gene of *Streptococcus pneumoniae*, J Bacteriol (vol. 170), No.

12, pp. 5931-4. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=2903859

[102] Abeyta, M.; Hardy, G. G. and Yother, J. (2003): Genetic alteration of capsule type but not PspA type affects accessibility of surface-bound complement and surface antigens of *Streptococcus pneumoniae*, Infect Immun (vol. 71), No. 1, pp. 218-25. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12496169

[103] Janulczyk, R.; Iannelli, F.; Sjoholm, A. G.; Pozzi, G. and Bjorck, L. (2000): Hic, a novel surface protein of *Streptococcus pneumoniae* that interferes with complement function, J Biol Chem (vol. 275), No. 47, pp. 37257-63. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10967103

[104] Crisostomo, M. I.; Vollmer, W.; Kharat, A. S.; Inhulsen, S.; Gehre, F.; Buckenmaier, S. and Tomasz, A. (2006): Attenuation of penicillin resistance in a peptidoglycan O-acetyl transferase mutant of *Streptococcus pneumoniae*, Mol Microbiol (vol. 61), No. 6, pp. 1497-509. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16968223

[105] Bera, A.; Herbert, S.; Jakob, A.; Vollmer, W. and Gotz, F. (2005): Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan *O*-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*., Mol. Microbiol. (vol. 55), pp. 778-787. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15661003

[106] Hakenbeck, R.; Madhour, A.; Denapaite, D. and Bruckner, R. (2009): Versatility of choline metabolism and choline-binding proteins in *Streptococcus pneumoniae* and commensal streptococci, FEMS Microbiol Rev (vol. 33), No. 3, pp. 572-86. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19396958

[107] Reichmann, P.; Konig, A.; Linares, J.; Alcaide, F.; Tenover, F. C.; McDougal, L.; Swidsinski, S. and Hakenbeck, R. (1997): A global gene pool for high-level cephalosporin resistance in commensal Streptococcus species and *Streptococcus pneumoniae*, J Infect Dis (vol. 176), No. 4, pp. 1001-12. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9333159

[108] Hoskins, J.; Alborn, W. E., Jr.; Arnold, J.; Blaszczak, L. C.; Burgett, S.; DeHoff, B. S.; Estrem, S. T.; Fritz, L.; Fu, D. J.; Fuller, W.; Geringer, C.; Gilmour, R.; Glass, J. S.; Khoja, H.; Kraft, A. R.; Lagace, R. E.; LeBlanc, D. J.; Lee, L. N.; Lefkowitz, E. J.; Lu, J.; Matsushima, P.; McAhren, S. M.; McHenney, M.; McLeaster, K.; Mundy, C. W.; Nicas, T. I.; Norris, F. H.; O'Gara, M.; Peery, R. B.; Robertson, G. T.; Rockey, P.; Sun, P. M.; Winkler, M. E.; Yang, Y.; Young-Bellido, M.; Zhao, G.; Zook, C. A.; Baltz, R. H.; Jaskunas, S. R.; Rosteck, P. R., Jr.; Skatrud, P. L. and Glass, J. I. (2001): Genome of the bacterium *Streptococcus pneumoniae* strain R6, J Bacteriol (vol. 183), No. 19, pp. 5709-17. URL:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11544234

[109] Leib, S. L.; Leppert, D.; Clements, J. and Tauber, M. G. (2000): Matrix metalloproteinases contribute to brain damage in experimental pneumococcal meningitis, Infect Immun (vol. 68), No. 2, pp. 615-20. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10639424

[110] Leib, S. L.; Kim, Y. S.; Chow, L. L.; Sheldon, R. A. and Tauber, M. G. (1996): Reactive oxygen intermediates contribute to necrotic and apoptotic neuronal injury in an infant rat model of bacterial meningitis due to group B streptococci, J Clin Invest (vol. 98), No. 11, pp. 2632-9. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8958228

[111] Gianinazzi, C.; Grandgirard, D.; Imboden, H.; Egger, L.; Meli, D. N.; Bifrare, Y. D.; Joss, P. C.; Tauber, M. G.; Borner, C. and Leib, S. L. (2003): Caspase-3 mediates hippocampal apoptosis in pneumococcal meningitis, Acta Neuropathol (Berl) (vol. 105), No. 5, pp. 499-507. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12677451

[112] Grandgirard, D.; Bifrare, Y. D.; Pleasure, S. J.; Kummer, J.; Leib, S. L. and Tauber, M. G. (2007): Pneumococcal meningitis induces apoptosis in recently postmitotic immature neurons in the dentate gyrus of neonatal rats, Dev Neurosci (vol. 29), No. 1-2, pp. 134-42. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17148956

[113] Dinarello, C. A. (2000): Proinflammatory cytokines, Chest (vol. 118), No. 2, pp. 503-8. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10936147

[114] Tang, T.; Frenette, P. S.; Hynes, R. O.; Wagner, D. D. and Mayadas, T. N. (1996): Cytokine-induced meningitis is dramatically attenuated in mice deficient in endothelial selectins, J Clin Invest (vol. 97), No. 11, pp. 2485-90. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8647940

[115] Barone, F. C.; Hillegass, L. M.; Price, W. J.; White, R. F.; Lee, E. V.; Feuerstein, G. Z.; Sarau, H. M.; Clark, R. K. and Griswold, D. E. (1991): Polymorphonuclear leukocyte infiltration into cerebral focal ischemic tissue: myeloperoxidase activity assay and histologic verification, J Neurosci Res (vol. 29), No. 3, pp. 336-45. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=1656059

[116] Barone, F. C.; Hillegass, L. M.; Tzimas, M. N.; Schmidt, D. B.; Foley, J. J.; White, R. F.; Price, W. J.; Feuerstein, G. Z.; Clark, R. K.; Griswold, D. E. and et al. (1995): Time-related changes in myeloperoxidase activity and leukotriene B4 receptor binding reflect leukocyte influx in cerebral focal stroke, Mol Chem Neuropathol (vol. 24), No. 1, pp. 13-30. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7755844

References

[117] Hansbrough, J. F.; Wikstrom, T.; Braide, M.; Tenenhaus, M.; Rennekampff, O. H.; Kiessig, V. and Bjursten, L. M. (1996): Neutrophil activation and tissue neutrophil sequestration in a rat model of thermal injury, J Surg Res (vol. 61), No. 1, pp. 17-22. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8769936

[118] Segal, A. W. (2005): How neutrophils kill microbes, Annu Rev Immunol (vol. 23), pp. 197-223. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15771570

[119] Weber, J. R.; Moreillon, P. and Tuomanen, E. I. (2003): Innate sensors for Gram-positive bacteria, Curr Opin Immunol (vol. 15), No. 4, pp. 408-15. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12900272

[120] Schwandner, R.; Dziarski, R.; Wesche, H.; Rothe, M. and Kirschning, C. J. (1999): Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2, J Biol Chem (vol. 274), No. 25, pp. 17406-9. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10364168

[121] Schroder, N. W.; Morath, S.; Alexander, C.; Hamann, L.; Hartung, T.; Zahringer, U.; Gobel, U. B.; Weber, J. R. and Schumann, R. R. (2003): Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved, J Biol Chem (vol. 278), No. 18, pp. 15587-94. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12594207

[122] Bera, A.; Herbert, S.; Jakob, A.; Vollmer, W. and Gotz, F. (2005): Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*, Mol Microbiol (vol. 55), No. 3, pp. 778-87. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15661003

[123] York, L., J. (1997): Enzymes: Classification, Kinetics, and Control, Devlin T., M., Textbook of Biochemistry with Clinical Correlations, Wiley-Liss, Inc., New York.

[124] Davis, K. M.; Akinbi, H. T.; Standish, A. J. and Weiser, J. N. (2008): Resistance to mucosal lysozyme compensates for the fitness deficit of peptidoglycan modifications by *Streptococcus pneumoniae*, PLoS Pathog (vol. 4), No. 12, p. e1000241. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19079576

[125] Horne, D. S. and Tomasz, A. (1993): Possible role of a choline-containing teichoic acid in the maintenance of normal cell shape and physiology in *Streptococcus oralis*, J Bacteriol (vol. 175), No. 6, pp. 1717-22. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8449879

[126] Swiatlo, E; McDaniel, LS and Briles, DE (2004): Choline binding proteins., Tuomanen, EI, Mitchell, TJ, Morrison, DA and Spratt, BG, In The Pneumococcus pp. 49-60, ASM, Washington, DC.

[127] Draing, C.; Pfitzenmaier, M.; Zummo, S.; Mancuso, G.; Geyer, A.; Hartung, T. and von Aulock, S. (2006): Comparison of lipoteichoic acid from different serotypes of *Streptococcus pneumoniae*, J Biol Chem (vol. 281), No. 45, pp. 33849-59. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16943191

[128] Fillon, S.; Soulis, K.; Rajasekaran, S.; Benedict-Hamilton, H.; Radin, J. N.; Orihuela, C. J.; El Kasmi, K. C.; Murti, G.; Kaushal, D.; Gaber, M. W.; Weber, J. R.; Murray, P. J. and Tuomanen, E. I. (2006): Platelet-activating factor receptor and innate immunity: uptake of gram-positive bacterial cell wall into host cells and cell-specific pathophysiology, J Immunol (vol. 177), No. 9, pp. 6182-91. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17056547

[129] Tuomanen, E.; Liu, H.; Hengstler, B.; Zak, O. and Tomasz, A. (1985): The induction of meningeal inflammation by components of the pneumococcal cell wall, J Infect Dis (vol. 151), No. 5, pp. 859-68. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=3989321

[130] Orihuela, C. J.; Fillon, S.; Smith-Sielicki, S. H.; El Kasmi, K. C.; Gao, G.; Soulis, K.; Patil, A.; Murray, P. J. and Tuomanen, E. I. (2006): Cell wall-mediated neuronal damage in early sepsis, Infect Immun (vol. 74), No. 7, pp. 3783-9. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16790750

[131] Poyart, C.; Pellegrini, E.; Marceau, M.; Baptista, M.; Jaubert, F.; Lamy, M. C. and Trieu-Cuot, P. (2003): Attenuated virulence of *Streptococcus agalactiae* deficient in D-alanyl-lipoteichoic acid is due to an increased susceptibility to defensins and phagocytic cells, Mol Microbiol (vol. 49), No. 6, pp. 1615-25. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12950925

[132] Du Clos, T. W. and Mold, C. (2001): The role of C-reactive protein in the resolution of bacterial infection, Curr Opin Infect Dis (vol. 14), No. 3, pp. 289-93. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11964845

[133] Scott, M. G.; Briles, D. E.; Shackelford, P. G.; Smith, D. S. and Nahm, M. H. (1987): Human antibodies to phosphocholine. IgG anti-PC antibodies express restricted numbers of V and C regions, J Immunol (vol. 138), No. 10, pp. 3325-31. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=3571975

[134] McDaniel, L. S.; Benjamin, W. H., Jr.; Forman, C. and Briles, D. E. (1984): Blood clearance by anti-phosphocholine antibodies as a mechanism of protec-

tion in experimental pneumococcal bacteremia, J Immunol (vol. 133), No. 6, pp. 3308-12. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=6491288

[135] Peschel, A. and Sahl, H. G. (2006): The co-evolution of host cationic antimicrobial peptides and microbial resistance, Nat Rev Microbiol (vol. 4), No. 7, pp. 529-36. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16778838

[136] Goldstein, B. P.; Wei, J.; Greenberg, K. and Novick, R. (1998): Activity of nisin against *Streptococcus pneumoniae*, in vitro, and in a mouse infection model, J Antimicrob Chemother (vol. 42), No. 2, pp. 277-8. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9738856

[137] Lysenko, E. S.; Gould, J.; Bals, R.; Wilson, J. M. and Weiser, J. N. (2000): Bacterial phosphorylcholine decreases susceptibility to the antimicrobial peptide LL-37/hCAP18 expressed in the upper respiratory tract, Infect Immun (vol. 68), No. 3, pp. 1664-71. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10678986

[138] Tanaka, N.; Fukuyama, S.; Fukuiwa, T.; Kawabata, M.; Sagara, Y.; Ito, H. O.; Miwa, Y.; Nagatake, T.; Kiyono, H. and Kurono, Y. (2007): Intranasal immunization with phosphorylcholine induces antigen specific mucosal and systemic immune responses in mice, Vaccine (vol. 25), No. 14, pp. 2680-7. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17270319

[139] Fischer, H. and Tomasz, A. (1984): Production and release of peptidoglycan and wall teichoic acid polymers in pneumococci treated with beta-lactam antibiotics, J Bacteriol (vol. 157), No. 2, pp. 507-13. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=6693350

[140] Ren, B.; McCrory, M. A.; Pass, C.; Bullard, D. C.; Ballantyne, C. M.; Xu, Y.; Briles, D. E. and Szalai, A. J. (2004): The virulence function of *Streptococcus pneumoniae* surface protein A involves inhibition of complement activation and impairment of complement receptor-mediated protection, J Immunol (vol. 173), No. 12, pp. 7506-12. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15585877

[141] Gosink, K. K.; Mann, E. R.; Guglielmo, C.; Tuomanen, E. I. and Masure, H. R. (2000): Role of novel choline binding proteins in virulence of *Streptococcus pneumoniae*, Infect Immun (vol. 68), No. 10, pp. 5690-5. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10992472

[142] Rosenow, C.; Ryan, P.; Weiser, J. N.; Johnson, S.; Fontan, P.; Ortqvist, A. and Masure, H. R. (1997): Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*, Mol Microbiol (vol. 25), No. 5, pp. 819-29. URL:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9364908

[143] Axelrod, S.; Oschkinat, H.; Enders, J.; Schlegel, B.; Brinkmann, V.; Kaufmann, S. H.; Haas, A. and Schaible, U. E. (2008): Delay of phagosome maturation by a mycobacterial lipid is reversed by nitric oxide, Cell Microbiol (vol. 10), No. 7, pp. 1530-45. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18363878

[144] Garcia-Bustos, J. F.; Chait, B. T. and Tomasz, A. (1988): Altered peptidoglycan structure in a pneumococcal transformant resistant to penicillin., J. Bacteriol. (vol. 170), pp. 2143-2147. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=3360741

[145] Severin, A. and Tomasz, A. (1996): Naturally occurring peptidoglycan variants of *Streptococcus pneumoniae.*, J. Bacteriol. (vol. 178), pp. 168-174. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8550412

[146] Filipe, S. R. and Tomasz, A. (2000): Inhibition of the expression of penicillin resistance in *Streptococcus pneumoniae* by inactivation of cell wall muropeptide branching genes., Proc. Natl. Acad. Sci. U S A (vol. 97), pp. 4891-4896. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10759563

[147] Meli, D. N.; Coimbra, R. S.; Erhart, D. G.; Loquet, G.; Bellac, C. L.; Tauber, M. G.; Neumann, U. and Leib, S. L. (2006): Doxycycline reduces mortality and injury to the brain and cochlea in experimental pneumococcal meningitis, Infect Immun (vol. 74), No. 7, pp. 3890-6. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16790761

[148] Sellner, J. and Leib, S. L. (2006): In bacterial meningitis cortical brain damage is associated with changes in parenchymal MMP-9/TIMP-1 ratio and increased collagen type IV degradation, Neurobiol Dis (vol. 21), No. 3, pp. 647-56. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16257222

[149] Bifrare, Y. D.; Kummer, J.; Joss, P.; Tauber, M. G. and Leib, S. L. (2005): Brain-derived neurotrophic factor protects against multiple forms of brain injury in bacterial meningitis, J Infect Dis (vol. 191), No. 1, pp. 40-5. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15593001

[150] Gundersen, H. J. and Jensen, E. B. (1987): The efficiency of systematic sampling in stereology and its prediction, J Microsc (vol. 147), No. Pt 3, pp. 229-63. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=3430576

[151] Dhodapkar, K. M.; Kaufman, J. L.; Ehlers, M.; Banerjee, D. K.; Bonvini, E.; Koenig, S.; Steinman, R. M.; Ravetch, J. V. and Dhodapkar, M. V. (2005): Selective blockade of inhibitory Fcgamma receptor enables human dendritic cell maturation with IL-12p70 production and immunity to antibody-coated tumor

cells, Proc Natl Acad Sci U S A (vol. 102), No. 8, pp. 2910-5. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15703291

[152] Colino, J.; Shen, Y. and Snapper, C. M. (2002): Dendritic cells pulsed with intact *Streptococcus pneumoniae* elicit both protein- and polysaccharide-specific immunoglobulin isotype responses in vivo through distinct mechanisms, J Exp Med (vol. 195), No. 1, pp. 1-13. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11781360

[153] Sambrook, J; Fritsch, E.R. and Maniatis, T (1989): Molecular Cloning, A Laboratory Manual, Nolan, Chris, Ed, 2nd Edn. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, NY.

[154] Zighelboim, S. and Tomasz, A. (1980): Penicillin-binding proteins of multiply antibiotic-resistant South African strains of *Streptococcus pneumoniae*, Antimicrob Agents Chemother (vol. 17), No. 3, pp. 434-42. URL: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=6903436

Abbreviations

°C	Degree Celsius
^{3}H	Tritium
aa	Amino acid
AATGal	D-2-acetamido-4-amino-2,4,6-trideoxygalactose
Adr	Attenuator of drug resistance
AIDS	Acquired immunodeficiency syndrom
amu	Atomic mass unit
ATP	Adenosin tri-phosphate
BAP	Blood agar plate
$CaCl_2$	Calcium chloride
CAMPs	Cationic antimicrobial peptides
CBP	Choline-binding protein
CD	Cluster of differentiation
CDP	Cytidine di-phosphate
CFU	Colony-forming unit
Cho$^-$	Choline-independent strain
CO_2	Carbon dioxide
CpG	Cytosin-phosphate-guanine
C-polysaccharide	Synonym for wall teichoic acid
Cps	Capsular polysaccharide synthesis locus
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CTP	Cytidine tri-phosphate
D-Ala	D-Alanine
DC	Dendritic cell
D-iGln	D-isoglutamine
DNA	Desoxyribonucleic acid
DOC	Deoxycholate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
F-Antigen	Forssman-Antigen (synomym for LTA)
g	Gravitational constant

Abbreviations

Gal	Galactose
Gal*N*Ac	D-N-acetyl-galactosamine
Glc	D-Glucose
Glc-Acyl$_2$-Gro	Monoglucosyldiacylglycerol
Glc*N*Ac	N-acetyl-glucosamine
GM-CSF	Granulocyte macrophage colony stimulating factor
h	Hour
HCl	Hydrochloric acid
HETAB	Hexadecyltrimethylammonium bromide
Hic	Factor H-binding inhibitor of complement
HNP1-3	Human neutrophil peptide
HPLC	High performance liquid chromatography
i.c.	Intracisternal
i.p.	Intraperitoneal
ICAM	Inter-cellular adhesion molecule
IFN-γ	Interferon-γ
IgA/M/G	Immunoglobuline A/M/G
IL	Interleukin
l	Liter
L-Ala	L-Alanine
L-Lys	L-Lysine
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
M	Molarity
MAC	Membrane attack complex
MARCO	Macrophage receptor with collagenous structure
MBL	Mannose-binding lectin
mg	Milligram
MHC	Major histocompatibility complex
min	Minute
ml	Milliliter
mM	Millimolar
mm^2	Square millimeter
MMP	Matrixmetalloproteinase

MPO	Myeloperoxidase
Mur*N*Ac	N-acetyl-muramic acid
NaCl	Sodium-chloride
NADPH	Nicotineamide adenine dinucleotide phosphate
NanA	Neuraminidase A
NETs	Neutrophil extracellular traps
ng	Nanogram
nm	Nanometer
Nod	Nucleotide oligomerization domain
OD	Optical density
p.i.	Post infection
Paf	Platelet-activating factor
PAMP	Pathogen associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PBP	Penicillin-binding protein
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PgdA	Peptidoglycan-*N*-acetyl-glucosamine-deacetylase
PGRP-L/S	Peptidoglycan-recognition protein-L/S
PiaA	Pneumococcal iron acquisition A
PiuA	Pneumococcal iron uptake A
PPIase	Peptidyl-prolyl-isomerase
PRR	Pattern-recognition receptor
PsaA	Pneumococcal Surface Adhesin A
rCRAMP	Rat cathelicidin-related antimicrobial peptide
Rit-P	Ribitol-5-phosphate
rPaf	Receptor for Platelet-activating factor
SAP	Serum amyloid protein
SDS	Sodium-dodecyl sulfate
Sec	Second
SrtA/B/C/D	Sortase A/B/C/D
TacF	Teichoic acid flippase
TI	T-cell independent
TLR	Toll-like receptor

Abbreviations

TNFα	Tumor-necrosis factor α
Tris	Tris(hydroxymethyl)aminomethane
TSA	Tryptic soy agar
U	Unit
UDP	Undecaprenylphosphate
UV	Ultraviolet
V	Volt
WBC	White blood cell
WHO	World Health Organization
WTA	Wall teichoic acid
β-Gal	β-Galactosidase
µCi	MicroCurie
µg	Microgram
µl	Microliter

Acknowledgements

First of all I would like to thank Dr. Tomasz for giving me the great opportunity to work in his lab. With all his enthusiasm, knowledge and experience he showed me how exciting the work on pneumo can be. Thanks to him, I learned how to approach and solve scientific problems and I feel very well prepared for my future. Besides being my mentor in the lab, Dr. Tomasz was always very helpful and understanding whenever private problems came up during my stay. I am especially grateful for the brief introduction to the Hungarian language I received. Taken together it was definitely also because of Dr. Tomasz that made my stay in New York a more than enjoyable experience.

I also would like to thank Prof. Hamann for being my Doktorvater at the Humboldt-University and for supporting me from Germany during my thesis work in the US.

A special thanks also goes to Dr. Waldemar Vollmer who gave me the opportunity to begin the work on my thesis in his former lab in Tübingen. These three months were already a perfect start and I obtained all the biochemical knowledge, necessary to work on the cell wall of bacteria. During my whole thesis, Waldemar always critically followed and supervised my work and read and improved my manuscripts.

I also want to thank current and former members of the lab. In particular I would like to thank Aude and Sandro, who actually became very good friends. Also Marie who helped me a lot with all the organization at Rockefeller and so many other things. And Arun who taught me how to work with pneumo. And Nelson, Marilyn, Halina, Susana, Herminia de Lencastre, Michael and Choon who all contributed to the fact that I really enjoyed the time I spent in the lab and in New York.

I want to thank my parents who always supported me during all the time.

Finally I would like to thank my girlfriend Sara - obrigado.

Publications and Oral Presentations

Gehre F, Spisek R, Kharat AS, Matthews P, Kukreja A, Dhodapkar MV, Tomasz A. The role of teichoic acid choline moieties in the virulence of *Streptococcus pneumoniae*. Accepted in Infect. Immun. 2009

Kharat AS, Denapaite D, **Gehre F**, Brückner R, Vollmer W, Hakenbeck R, Tomasz A. Different pathways of choline metabolism in two choline-independent strains of *Streptococcus pneumoniae* and their impact on virulence. J Bacteriol. 2008 Sep;190(17):5907-14.

Gehre F, Leib SL, Grandgirard D, Kummer J, Buehlmann A, Simon F, Gäumann R, Kharat AS, Täuber MG, Tomasz A. Essential role of choline for pneumococcal virulence in an experimental model of meningitis. J Intern Med. 2008 Aug; 264(2): 143-54.

Klotz C, **Gehre F**, Lucius R, Pogonka T. Identification of *Eimeria tenella* genes encoding for secretory proteins and evaluation of candidates by DNA immunisation studies in chickens. Vaccine. 2007 Sep 4; 25(36):6625-34.

Crisóstomo MI, Vollmer W, Kharat AS, Inhülsen S, **Gehre F**, Buckenmaier S, Tomasz A. Attenuation of penicillin resistance in a peptidoglycan O-acetyl transferase mutant of *Streptococcus pneumoniae*. Mol Microbiol. 2006 Sep; 61(6):1497-509.

Schwenkenbecher JM, Frohlich C, **Gehre F**, Schnur LF, Schonian G. Evolution and conservation of microsatellite markers for *Leishmania tropica*. Infect Genet Evol. 2004 Jun; 4(2):99-105.

Essential role of choline for pneumococcal virulence in an experimental model of meningitis. Oral Presentation, Europneumo 2007- 8th European Meeting on the Molecular Biology of the Pneumococcus, Oeiras, Portugal

Teichoic acid genes and the role of choline in pneumococcal virulence. Invited speaker, ISPPD 2008, 6th International Symposium on Pneumococci & Pneumococcal Diseases, Reykjavik, Iceland

Eidestattliche Erklärung

Ich versichere, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.